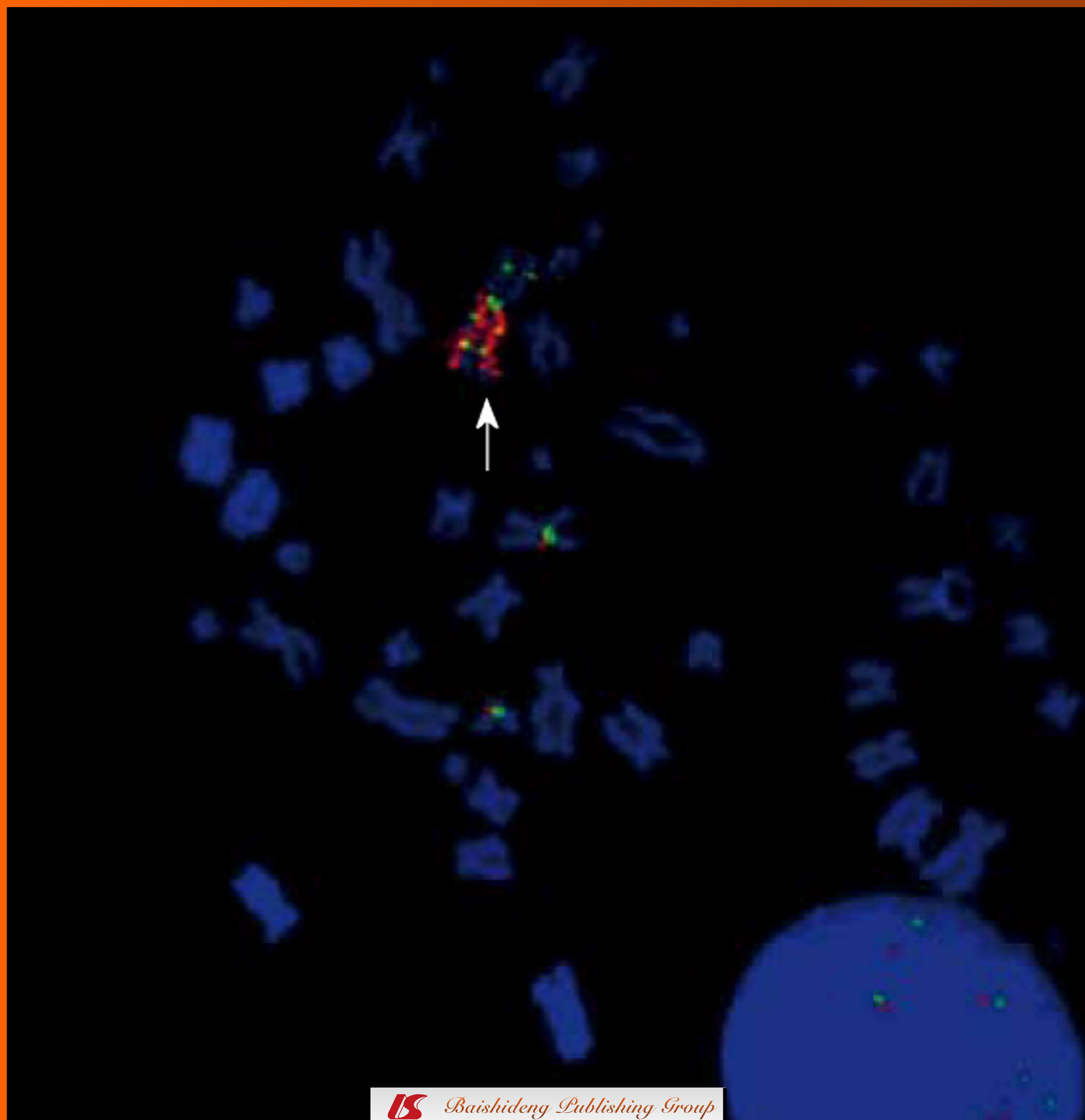
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2011 June 28; 17(24): 2879-2976



Baishideng Publishing Group
www.wjgnet.com

WJG

World Journal of Gastroenterology

A peer-reviewed, online, open-access journal of gastroenterology and hepatology

Editorial Board

2010-2013

The *World Journal of Gastroenterology* Editorial Board consists of 1144 members, representing a team of worldwide experts in gastroenterology and hepatology. They are from 60 countries, including Albania (1), Argentina (8), Australia (29), Austria (14), Belgium (12), Brazil (10), Brunei Darussalam (1), Bulgaria (2), Canada (20), Chile (3), China (69), Colombia (1), Croatia (2), Cuba (1), Czech (4), Denmark (8), Ecuador (1), Egypt (2), Estonia (2), Finland (8), France (24), Germany (75), Greece (14), Hungary (10), India (26), Iran (6), Ireland (7), Israel (12), Italy (101), Japan (112), Jordan (1), Kuwait (1), Lebanon (3), Lithuania (2), Malaysia (1), Mexico (10), Moldova (1), Netherlands (29), New Zealand (2), Norway (11), Pakistan (2), Poland (11), Portugal (4), Romania (3), Russia (1), Saudi Arabia (3), Serbia (3), Singapore (10), South Africa (2), South Korea (32), Spain (38), Sweden (18), Switzerland (11), Thailand (1), Trinidad and Tobago (1), Turkey (24), United Arab Emirates (2), United Kingdom (82), United States (249), and Uruguay (1).

HONORARY EDITORS-IN-CHIEF
James L Boyer, *New Haven*
Ke-Ji Chen, *Beijing*
Martin H Floch, *New Haven*
Emmet B Keeffe, *Palo Alto*
Geng-Tao Liu, *Beijing*
Lein-Ray Mo, *Tainan*
Eamonn M Quigley, *Cork*
Rafiq A Sheikh, *Sacramento*
Nicholas J Talley, *Rochester*
Ming-Lung Yu, *Kaohsiung*

PRESIDENT AND EDITOR-IN-CHIEF
Lian-Sheng Ma, *Beijing*

ACADEMIC EDITOR-IN-CHIEF
Tauseef Ali, *Oklahoma City*
Mauro Bortolotti, *Bologna*
Tarkan Karakan, *Ankara*
Weekitt Kittisupamongkol, *Bangkok*
Anastasios Koulaouzidis, *Edinburgh*
Bo-Rong Pan, *Xi'an*
Sylvia LF Pender, *Southampton*
Max S Petrov, *Auckland*
George Y Wu, *Farmington*

STRATEGY ASSOCIATE EDITORS-IN-CHIEF
Peter Draganov, *Florida*
Hugh J Freeman, *Vancouver*
Maria C Gutiérrez-Ruiz, *Mexico*
Kazuhiro Hanazaki, *Kochi*
Akio Inui, *Kagoshima*
Kalpesh Jani, *Baroda*
Javier S Martin, *Punta del Este*
Natalia A Osna, *Omaha*
Wei Tang, *Tokyo*
Alan BR Thomson, *Edmonton*
Harry HX Xia, *Hanover*
Jesus K Yamamoto-Furusho, *Mexico*
Yoshio Yamaoka, *Houston*

ASSOCIATE EDITORS-IN-CHIEF
You-Yong Lu, *Beijing*
John M Luk, *Singapore*
Hiroshi Shimada, *Yokohama*

GUEST EDITORIAL BOARD MEMBERS
Chien-Jen Chen, *Taipei*
Yang-Yuan Chen, *Changhua*
Jen-Hwey Chiu, *Taipei*
Seng-Kee Chuah, *Kaohsiung*
Wan-Long Chuang, *Kaohsiun*
Ming-Chih Hou, *Taipei*
Kevin Cheng-Wen Hsiao, *Taipei*
Po-Shiuan Hsieh, *Taipei*
Tsung-Hui Hu, *Kaohsiung*
Wen-Hsin Huang, *Taichung*
Chao-Hung Hung, *Kaohsiung*
I-Rue Lai, *Taipei*
Teng-Yu Lee, *Taichung*
Ching Chung Lin, *Taipei*
Hui-Kang Liu, *Taipei*
Hon-Yi Shi, *Kaohsiung*
Chih-Chi Wang, *Kaohsiung*
Jin-Town Wang, *Taipei*
Cheng-Shyong Wu, *Chia-Yi*
Jaw-Ching Wu, *Taipei*
Jiunn-Jong Wu, *Tainan*
Ming-Shiang Wu, *Taipei*
Ta-Sen Yeh, *Taoyuan*
Hsu-Heng Yen, *Changhua*
Ming-Whei Yu, *Taipei*

MEMBERS OF THE EDITORIAL BOARD

Albania
Bashkim Resuli, *Tirana*

Argentina
Julio H Carri, *Córdoba*
Eduardo de Santibañes, *Buenos Aires*
Bernardo Frider, *Buenos Aires*
Carlos J Pirola, *Buenos Aires*
Bernabe Matias Quesada, *Buenos Aires*
Silvia Sookoian, *Buenos Aires*
Adriana M Torres, *Rosario*
Maria Ines Vaccaro, *Buenos Aires*

Australia
Leon Anton Adams, *Nedlands*
Richard Anderson, *Victoria*
Minoti V Apte, *New South Wales*
Andrew V Biankin, *Sydney*
Filip Braet, *Sydney*
Christopher Christophi, *Melbourne*
Philip G Dinning, *Koagarah*
Guy D Eslick, *Sydney*
Michael A Fink, *Melbourne*

Robert JL Fraser, *Daw Park*
Jacob George, *Westmead*
Mark D Gorrell, *Sydney*
Alexander G Heriot, *Melbourne*
Michael Horowitz, *Adelaide*
John E Kellow, *Sydney*
William Kemp, *Melbourne*
Finlay A Macrae, *Victoria*
Daniel Markovich, *Brisbane*
Vance Matthews, *Melbourne*
Phillip S Oates, *Perth*
Shan Rajendra, *Tasmania*
Rajvinder Singh, *Elizabeth Vale*
Ross C Smith, *Sydney*
Kevin J Spring, *Brisbane*
Nathan Subramaniam, *Brisbane*
Phil Sutton, *Melbourne*
Cuong D Tran, *North Adelaide*
Debbie Trinder, *Fremantle*
David Ian Watson, *Bedford Park*

Austria

Herwig R Cerwenka, *Graz*
Ashraf Dahaba, *Graz*
Peter Ferenci, *Vienna*
Valentin Fuhrmann, *Vienna*
Alfred Gangl, *Vienna*
Alexander M Hirschl, *Wien*
Kurt Lenz, *Linz*
Dietmar Öfner, *Salzburg*
Markus Peck-Radosavljevic, *Vienna*
Markus Raderer, *Vienna*
Stefan Riss, *Vienna*
Georg Roth, *Vienna*
Michael Trauner, *Graz*
Thomas Wild, *Kapellerfeld*

Belgium

Rudi Beyaert, *Gent*
Benedicte Y De Winter, *Antwerp*
Inge I Depoortere, *Leuven*
Olivier Detry, *Liège*
Philip Meuleman, *Ghent*
Marc Peeters, *De Pintelaan*
Freddy Penninckx, *Leuven*
Jean-Yves L Reginster, *Liège*
Mark De Ridder, *Brussels*
Etienne M Sokal, *Brussels*
Kristin Verbeke, *Leuven*
Eddie Wisse, *Keerbergen*

Brazil

José LF Caboclo, *São José do Rio Preto*
Roberto J Carvalho-Filho, *São Paulo*
Jaime Natan Eisig, *São Paulo*
Andre Castro Lyra, *Salvador*
Marcelo Lima Ribeiro, *Braganca Paulista*
Joao Batista Teixeira Rocha, *Santa Maria*
Heitor Rosa, *Goiania*
Damiao C Moraes Santos, *Rio de Janeiro*
Ana Cristina Simões e Silva, *Belo Horizonte*
Eduardo Garcia Vilela, *Belo Horizonte*

Brunei Darussalam

Vui Heng Chong, *Bandar Seri Begawan*

Bulgaria

Zahariy Krastev, *Sofia*
Mihaela Petrova, *Sofia*

Canada

Alain Bitton, *Montreal*
Michael F Byrne, *Vancouver*
Kris Chadee, *Calgary*
Wangxue Chen, *Ottawa*
Ram Prakash Galwa, *Ottawa*
Philip H Gordon, *Montreal*
Waliul Khan, *Ontario*
Qiang Liu, *Saskatoon*
John K Marshall, *Ontario*
Andrew L Mason, *Alberta*
Kostas Pantopoulos, *Quebec*
Nathalie Perreault, *Sherbrooke*
Baljinder Singh Salh, *Vancouver*
Eldon Shaffer, *Calgary*
Martin Storr, *Calgary*
Pingchang Yang, *Hamilton*
Eric M Yoshida, *Vancouver*
Claudia Zwingmann, *Montreal*

Chile

Marcelo A Beltran, *La Serena*
Xabier De Aretxabala, *Santiago*
Silvana Zanlungo, *Santiago*

China

Hui-Jie Bian, *Xi'an*
San-Jun Cai, *Shanghai*
Guang-Wen Cao, *Shanghai*
Xiao-Ping Chen, *Wuhan*
Chi-Hin Cho, *Hong Kong*
Zong-Jie Cui, *Beijing*
Jing-Yuan Fang, *Shanghai*
De-Liang Fu, *Shanghai*
Ze-Guang Han, *Shanghai*
Chun-Yi Hao, *Beijing*
Ming-Liang He, *Hong Kong*
Ching-Lung Lai, *Hong Kong*
Simon Law, *Hong Kong*
Yuk-Tong Lee, *Hong Kong*
En-Min Li, *Shantou*
Fei Li, *Beijing*
Yu-Yuan Li, *Guangzhou*
Zhao-Shen Li, *Shanghai*
Xing-Hua Lu, *Beijing*
Yi-Min Mao, *Shanghai*
Qin Su, *Beijing*
Paul Kwong-Hang Tam, *Hong Kong*
Yuk Him Tam, *Hong Kong*
Ren-Xiang Tan, *Nanjing*
Wei-Dong Tong, *Chongqing*
Eric WC Tse, *Hong Kong*
Fu-Sheng Wang, *Beijing*
Xiang-Dong Wang, *Shanghai*
Nathalie Wong, *Hong Kong*
Justin CY Wu, *Hong Kong*
Wen-Rong Xu, *Zhenjiang*
An-Gang Yang, *Xi'an*
Wei-Cheng You, *Beijing*
Chun-Qing Zhang, *Jinan*
Jian-Zhong Zhang, *Beijing*
Xiao-Peng Zhang, *Beijing*
Xuan Zhang, *Beijing*

Colombia

Germán Campuzano-Maya, *Medellín*

Croatia

Tamara Cacev, *Zagreb*
Marko Duvnjak, *Zagreb*

Cuba

Damian C Rodriguez, *Havana*

Czech

Jan Bures, *Hradec Kralove*
Milan Jirsa, *Praha*
Marcela Kopacova, *Hradec Kralove*
Pavel Trunečka, *Prague*

Denmark

Leif Percival Andersen, *Copenhagen*
Asbjørn M Drewes, *Aalborg*
Morten Frisch, *Copenhagen*
Jan Mollenhauer, *Odense*
Morten Hylander Møller, *Holte*
Søren Rafaelsen, *Vejle*
Jorgen Rask-Madsen, *Skodsborg*
Peer Wille-Jørgensen, *Copenhagen*

Ecuador

Fernando E Sempértegui, *Quito*

Egypt

Zeinab Nabil Ahmed, *Cairo*
Hussein M Atta, *El-Minia*

Estonia

Riina Salupere, *Tartu*
Tamara Vorobjova, *Tartu*

Finland

Saila Kauhanen, *Turku*

Thomas Kietzmann, *Oulu*
Kaija-Leena Kolho, *Helsinki*
Jukka-Pekka Mecklin, *Jyvaskyla*
Minna Nyström, *Helsinki*
Pauli Antero Puolakkainen, *Turku*
Juhani Sand, *Tampere*
Lea Veijola, *Helsinki*

France

Claire Bonithon-Kopp, *Dijon*
Lionel Bueno, *Toulouse*
Sabine Colnot, *Paris*
Catherine Daniel, *Lille Cedex*
Alexis Desmoulière, *Limoges*
Thabut Dominique, *Paris*
Francoise L Fabiani, *Angers*
Jean-Luc Faucheron, *Grenoble*
Jean Paul Galmiche, *Nantes cedex*
Boris Guiu, *Dijon*
Paul Hofman, *Nice*
Laurent Huwart, *Paris*
Juan Iovanna, *Marseille*
Abdel-Majid Khatib, *Paris*
Philippe Lehours, *Bordeaux*
Flavio Maina, *Marseille*
Patrick Marcellin, *Paris*
Rene Gerolami Santandera, *Marseille*
Annie Schmid-Alliana, *Nice cedex*
Alain L Servin, *Châtenay-Malabry*
Stephane Supiot, *Nantes*
Baumert F Thomas, *Strasbourg*
Jean-Jacques Tuech, *Rouen*
Frank Zerbib, *Bordeaux Cedex*

Germany

Erwin Biecker, *Siegburg*
Hubert Blum, *Freiburg*
Thomas Bock, *Tuebingen*
Dean Bogoevski, *Hamburg*
Elfriede Bollschweiler, *Köln*
Jürgen Borlak, *Hannover*
Christa Buechler, *Regensburg*
Jürgen Büning, *Lübeck*
Elke Cario, *Essen*
Bruno Christ, *Halle/Saale*
Christoph F Dietrich, *Bad Mergentheim*
Ulrich R Fölsch, *Kiel*
Nikolaus Gassler, *Aachen*
Markus Gerhard, *Munich*
Dieter Glebe, *Giessen*
Ralph Graeser, *Freiburg*
Axel M Gressner, *Aachen*
Nils Habbe, *Marburg*
Thilo Hackert, *Heidelberg*
Wolfgang Hagmann, *Heidelberg*
Dirk Haller, *Freising*
Philip D Hard, *Giessen*
Claus Hellerbrand, *Regensburg*
Klaus R Herrlinger, *Stuttgart*
Eberhard Hildt, *Berlin*
Andrea Hille, *Goettingen*
Joerg C Hoffmann, *Berlin*
Philipe N Khalil, *Munich*
Andrej Khandoga, *Munich*
Jorg Kleeff, *Munich*
Ingmar Königsrainer, *Tübingen*
Peter Konturek, *Erlangen*
Stefan Kubicka, *Hannover*
Joachim Labenz, *Siegen*
Michael Linnebacher, *Rostock*
Jutta Elisabeth Lüttges, *Riegelsberg*
Peter Malfertheiner, *Magdeburg*
Oliver Mann, *Hamburg*
Peter N Meier, *Hannover*
Sabine Mihm, *Göttingen*
Klaus Mönkemüller, *Bottrop*
Jonas Mudter, *Erlangen*
Sebastian Mueller, *Heidelberg*
Robert Obermaier, *Freiburg*
Matthias Ocker, *Erlangen*
Stephan Johannes Ott, *Kiel*
Gustav Paumgartner, *Munich*
Christoph Reichel, *Bad Brückenau*
Markus Reiser, *Bochum*
Steffen Rickes, *Magdeburg*
Elke Roeb, *Giessen*
Christian Rust, *Munich*
Hans Scherubl, *Berlin*
Martin K Schilling, *Homburg*
Joerg F Schlaak, *Essen*
Rene Schmidt, *Freiburg*
Andreas G Schreyer, *Regensburg*
Karsten Schulmann, *Bochum*
Henning Schulze-Bergkamen, *Mainz*
Manfred V Singer, *Mannheim*
Jens Standop, *Bonn*
Jurgen M Stein, *Frankfurt*
Ulrike S Stein, *Berlin*
Wolfgang R Stremmel, *Heidelberg*
Harald F Teutsch, *Ulm*
Hans L Tillmann, *Leipzig*
Christian Trautwein, *Aachen*
Joerg Trojan, *Frankfurt*
Arndt Vogel, *Hannover*
Siegfried Wagner, *Deggendorf*
Frank Ulrich Weiss, *Greifswald*
Fritz von Weizsäcker, *Berlin*
Thomas Wex, *Magdeburg*
Stefan Wirth, *Wuppertal*
Marty Zdichavsky, *Tübingen*

Greece

Helen Christopoulou-Aletra, *Thessaloniki*
T Choli-Papadopoulou, *Thessaloniki*
Tsianos Epameinondas, *Ioannina*
Ioannis Kanellos, *Thessaloniki*
Elias A Kouroumalis, *Heraklion*
Ioannis E Koutroubakis, *Heraklion*
Michael Koutsilieris, *Athens*
Andreas Larentzakis, *Athens*
Emanuel K Manesis, *Athens*
Spilios Manolakopoulos, *Athens*
Konstantinos Mimidis, *Alexandroupolis*
George Papatheodoridis, *Athens*
Spiros Sgouros, *Athens*
Evangelos Tsiambas, *Ag Paraskevi Attiki*

Hungary

György M Buzás, *Budapest*
László Czakó, *Szeged*
Gyula Farkas, *Szeged*
Peter Hegyi, *Szeged*
Peter L Lakatos, *Budapest*
Yvette Mándi, *Szeged*
Zoltan Rakonczay, *Szeged*
Ferenc Sipos, *Budapest*
Zsuzsa Szondy, *Debrecen*
Gabor Veres, *Budapest*

India

Philip Abraham, *Mumbai*
Vineet Ahuja, *New Delhi*
Giriraj Ratan Chandak, *Hyderabad*
Devinder Kumar Dhawan, *Chandigarh*
Radha K Dhiman, *Chandigarh*
Pankaj Garg, *Panchkula*
Pramod Kumar Garg, *New Delhi*
Debidas Ghosh, *Midnpore*
Uday C Ghoshal, *Lucknow*
Bhupendra Kumar Jain, *Delhi*
Ashok Kumar, *Lucknow*
Bikash Medhi, *Chandigarh*
Sri P Misra, *Allahabad*
Gopal Nath, *Varanasi*
Samiran Nundy, *New Delhi*
Jagannath Palepu, *Mumbai*
Vandana Panda, *Mumbai*
Benjamin Perakath, *Tamil Nadu*
Ramesh Roop Rai, *Jaipur*
Nageshwar D Reddy, *Hyderabad*
Barjesh Chander Sharma, *New Delhi*
Virendra Singh, *Chandigarh*
Rupjyoti Talukdar, *Guwahati*
Rakesh Kumar Tandon, *New Delhi*
Jai Dev Wig, *Chandigarh*

Iran

Mohammad Abdollahi, *Tehran*
Peyman Adibi, *Isfahan*
Seyed-Moayed Alavian, *Tehran*
Seyed Mohsen Dehghani, *Shiraz*
Reza Malekzadeh, *Tehran*
Alireza Mani, *Tehran*

Ireland

Billy Bourke, *Dublin*
Ted Dinan, *Cork*
Catherine Greene, *Dublin*
Ross McManus, *Dublin*
Anthony P Moran, *Galway*
Marion Rowland, *Dublin*

Israel

Simon Bar-Meir, *Hashomer*
Alexander Becker, *Afula*
Abraham R Eliakim, *Haifa*
Sigal Fishman, *Tel Aviv*
Boris Kirshtein, *Beer Sheva*
Eli Magen, *Ashdod*
Menachem Moshkowitz, *Tel-Aviv*
Assy Nimer, *Safed*
Shmuel Odes, *Beer Sheva*
Mark Pines, *Bet Dagan*
Ron Shaoul, *Haifa*
Ami D Sperber, *Beer-Sheva*

Italy

Donato F Altomare, *Bari*
Piero Amodio, *Padova*
Angelo Andriulli, *San Giovanni Rotondo*
Paolo Angeli, *Padova*
Bruno Annibale, *Rome*
Paolo Aurello, *Rome*
Salvatore Auricchio, *Naples*
Antonio Basoli, *Rome*
Claudio Bassi, *Verona*
Gabrio Bassotti, *Perugia*
Mauro Bernardi, *Bologna*
Alberto Biondi, *Rome*
Luigi Bonavina, *Milano*
Guglielmo Borgia, *Naples*
Roberto Berni Canani, *Naples*
Maria Gabriella Caruso, *Bari*
Fausto Catena, *Bologna*
Giuseppe Chiarioni, *Valeggio*
Michele Cicala, *Rome*
Dario Conte, *Milano*
Francesco Costa, *Pisa*
Antonio Craxì, *Palermo*
Salvatore Cucchiara, *Rome*
Giuseppe Currò, *Messina*
Mario M D'Elios, *Florence*
Mirko D'Onofrio, *Verona*
Silvio Danese, *Milano*
Roberto de Franchis, *Milano*
Paola De Nardi, *Milan*
Giovanni D De Palma, *Naples*
Giuliana Decorti, *Trieste*
Gianlorenzo Dionigi, *Varese*
Massimo Falconi, *Verona*
Silvia Fargion, *Milan*
Giammarco Fava, *Ancona*
Francesco Feo, *Sassari*
Alessandra Ferlini, *Ferrara*
Alessandro Ferrero, *Torino*
Mirella Fraquelli, *Milan*
Luca Frulloni, *Verona*
Giovanni B Gaeta, *Napoli*
Antonio Gasbarrini, *Rome*
Edoardo G Giannini, *Genoa*
Alessandro Granito, *Bologna*
Fabio Grizzi, *Milan*
Salvatore Gruttadauria, *Palermo*
Pietro Invernizzi, *Milan*
Achille Iolascon, *Naples*
Angelo A Izzo, *Naples*
Ezio Laconi, *Cagliari*
Giovanni Latella, *L'Aquila*
Massimo Levrero, *Rome*
Francesco Luzza, *Catanzaro*
Lucia Malaguarnera, *Catania*
Francesco Manguso, *Napoli*
Pier Mannuccio Mannucci, *Milan*
Giancarlo Mansueto, *Verona*
Giulio Marchesini, *Bologna*
Mara Massimi, *Coppito*
Giovanni Milito, *Rome*
Giuseppe Montalto, *Palermo*
Giovanni Monteleone, *Rome*
Luca Morelli, *Trento*
Giovanni Musso, *Torino*
Mario Nano, *Torino*
Gerardo Nardone, *Napoli*
Riccardo Nascimbeni, *Brescia*
Valerio Nobili, *Rome*
Fabio Pace, *Milan*
Nadia Peparini, *Rome*
Marcello Persico, *Naples*
Mario Pescatori, *Rome*
Raffaele Pezzilli, *Bologna*
Alberto Piperno, *Monza*
Anna C Piscaglia, *Rome*
Piero Portincasa, *Bari*
Michele Reni, *Milan*
Vittorio Ricci, *Pavia*
Oliviero Riggio, *Rome*
Mario Rizzetto, *Torino*
Ballarin Roberto, *Modena*
Gerardo Rosati, *Potenza*
Franco Roviello, *Siena*
Cesare Ruffolo, *Treviso*
Massimo Rugge, *Padova*
Marco Scarpa, *Padova*
C armelo Scarpignato, *Parma*
Giuseppe Sica, *Rome*
Marco Silano, *Rome*
Pierpaolo Sileri, *Rome*
Vincenzo Stanghellini, *Bologna*
Fiorucci Stefano, *Perugia*
Giovanni Tarantino, *Naples*
Alberto Tommasini, *Trieste*
Guido Torzilli, *Rozzano Milan*
Cesare Tosetti, *Porretta Terme*
Antonello Trecca, *Rome*
Vincenzo Villanacci, *Brescia*
Lucia Ricci Vitiani, *Rome*
Marco Vivarelli, *Bologna*

Japan

Kyoichi Adachi, *Izumo*
Yasushi Adachi, *Sapporo*
Takafumi Ando, *Nagoya*
Akira Andoh, *Otsu*
Masahiro Arai, *Tokyo*
Hitoshi Asakura, *Tokyo*
Kazuo Chijiiwa, *Miyazaki*
Yuichiro Eguchi, *Saga*
Itaru Endo, *Yokohama*
Munechika Enjoji, *Fukuoka*
Yasuhiro Fujino, *Akashi*
Mitsuhiro Fujishiro, *Tokyo*
Kouhei Fukushima, *Sendai*
Masanori Hatakeyama, *Tokyo*
Keiji Hirata, *Kitakyushu*
Toru Hiyama, *Higashihiroshima*
Masahiro Iizuka, *Akita*
Susumu Ikehara, *Osaka*
Kenichi Ikejima, *Bunkyo-ku*
Yutaka Inagaki, *Kanagawa*
Hiromi Ishibashi, *Nagasaki*
Shunji Ishihara, *Izumo*
Toru Ishikawa, *Niigata*
Toshiyuki Ishiwata, *Tokyo*
Hajime Isomoto, *Nagasaki*
Yoshiaki Iwasaki, *Okayama*
Satoru Kakizaki, *Gunma*
Terumi Kamisawa, *Tokyo*
Mototsugu Kato, *Sapporo*
Naoya Kato, *Tokyo*
Takumi Kawaguchi, *Kurume*
Yohei Kida, *Kainan*
Shogo Kikuchi, *Aichi*
Tsuneo Kitamura, *Chiba*
Takashi Kobayashi, *Tokyo*
Yasuhiro Koga, *Isehara*
Takashi Kojima, *Sapporo*
Norihiro Kokudo, *Tokyo*
Masatoshi Kudo, *Osaka*
Shin Maeda, *Tokyo*
Satoshi Mamori, *Hyogo*
Atsushi Masamune, *Sendai*
Yasushi Matsuzaki, *Tsukuba*
Kenji Miki, *Tokyo*
Toshihiro Mitaka, *Sapporo*
Hiroto Miwa, *Hyogo*
Kotaro Miyake, *Tokushima*
Manabu Morimoto, *Yokohama*
Yoshiharu Motoo, *Kanazawa*
Yoshiaki Murakami, *Hiroshima*
Yoshiki Murakami, *Kyoto*
Kunihiko Murase, *Tusima*
Akihito Nagahara, *Tokyo*
Yuji Naito, *Kyoto*
Atsushi Nakajima, *Yokohama*
Hisato Nakajima, *Tokyo*
Hiroki Nakamura, *Yamaguchi*
Shotaro Nakamura, *Fukuoka*
Akimasa Nakao, *Nagogya*
Shuhei Nishiguchi, *Hyogo*
Mikio Nishioka, *Niihama*
Keiji Ogura, *Tokyo*
Susumu Ohmada, *Maebashi*
Hirohide Ohnishi, *Akita*
Kenji Okajima, *Nagoya*
Kazuichi Okazaki, *Osaka*
Morikazu Onji, *Ehime*
Satoshi Osawa, *Hamamatsu*
Hidetsugu Saito, *Tokyo*
Yutaka Saito, *Tokyo*
Naoaki Sakata, *Sendai*
Yasushi Sano, *Chiba*
Tokihiko Sawada, *Tochigi*
Tomohiko Shimatan, *Hiroshima*
Yukihiro Shimizu, *Kyoto*
Shinji Shimoda, *Fukuoka*
Yoshio Shirai, *Niigata*
Masayuki Sho, *Nara*
Shoichiro Sumi, *Kyoto*
Hidekazu Suzuki, *Tokyo*
Masahiro Tajika, *Nagoya*
Yoshihisa Takahashi, *Tokyo*
Toshinari Takamura, *Kanazawa*
Hiroaki Takeuchi, *Kochi*
Yoshitaka Takuma, *Okayama*
Akihiro Tamori, *Osaka*
Atsushi Tanaka, *Tokyo*
Shinji Tanaka, *Hiroshima*
Satoshi Tanno, *Hokkaido*
Shinji Togo, *Yokohama*
Hitoshi Tsuda, *Tokyo*
Hiroyuki Uehara, *Osaka*
Masahito Uemura, *Kashihara*
Yoshiyuki Ueno, *Sendai*
Mitsuyoshi Urashima, *Tokyo*
Takuya Watanabe, *Niigata*
Satoshi Yamagiwa, *Niigata*
Taketo Yamaguchi, *Chiba*
Mitsunori Yamakawa, *Yamagata*
Takayuki Yamamoto, *Yokkaichi*
Yutaka Yata, *Maebashi*
Hiroshi Yoshida, *Tokyo*
Norimasa Yoshida, *Kyoto*
Yuichi Yoshida, *Osaka*
Kentaro Yoshika, *Toyoake*
Hitoshi Yoshiji, *Nara*
Katsutoshi Yoshizato, *Higashihiroshima*
Tomoharu Yoshizumi, *Fukuoka*

Jordan

Ismail Matalka, *Irbid*

Kuwait

Islam Khan, *Safat*

Lebanon

Bassam N Abboud, *Beirut*
Ala I Sharara, *Beirut*
Rita Slim, *Beirut*

Lithuania

Giedrius Barauskas, *Kaunas*
Limas Kupcinskas, *Kaunas*

Malaysia

Andrew Seng Boon Chua, *Ipoh*

Mexico

Richard A Awad, *Mexico*
Aldo Torre Delgadillo, *Mexico*
Diego Garcia-Compean, *Monterrey*
Paulino M Hernández Magro, *Celaya*
Miguel Angel Mercado, *Distrito Federal*
Arturo Panduro, *Jalisco*
Omar Vergara-Fernandez, *Tlalpan*
Saúl Villa-Trevio, *Mexico*

Moldova

Igor Mishin, *Kishinev*

Netherlands

Ulrich Beuers, *Amsterdam*
Lee Bouwman, *Leiden*
Albert J Bredenoord, *Nieuwegein*
Lodewijk AA Brosens, *Utrecht*
J Bart A Crusius, *Amsterdam*
Wouter de Herder, *Rotterdam*
Pieter JF de Jonge, *Rotterdam*
Robert J de Knegt, *Rotterdam*
Wendy W Johanna de Leng, *Utrecht*
Annemarie de Vries, *Rotterdam*
James CH Hardwick, *Leiden*
Frank Hoentjen, *Haarlem*
Misha Luyer, *Sittard*
Jeroen Maljaars, *Maastricht*
Gerrit A Meijer, *Amsterdam*
Servaas Morré, *Amsterdam*
Chris JJ Mulder, *Amsterdam*
John Plukker, *Groningen*
Albert Frederik Pull ter Gunne, *Tilburg*
Paul E Sijens, *Groningen*
BW Marcel Spanier, *Arnhem*
Shiri Sverdlov, *Maastricht*
Maarten Tushuizen, *Amsterdam*
Jantine van Baal, *Heidelberglaan*
Astrid van der Velde, *The Hague*
Karel van Erpecum, *Utrecht*
Loes van Keimpema, *Nijmegen*
Robert Christiaan Verdonk, *Groningen*
Erwin G Zoetendal, *Wageningen*

New Zealand

Andrew S Day, *Christchurch*

Norway

Olav Dalgard, *Oslo*
Trond Peder Flaten, *Trondheim*
Reidar Fossmark, *Trondheim*
Rasmus Goll, *Tromso*
Ole Høie, *Arendal*
Asle W Medhus, *Oslo*
Espen Melum, *Oslo*
Trine Olsen, *Tromso*
Eyvind J Paulssen, *Tromso*
Jon Arne Søreide, *Stavanger*
Kjetil Soreide, *Stavanger*

Pakistan

Shahab Abid, *Karachi*
Syed MW Jafri, *Karachi*

Poland

Marek Bebenek, *Wroclaw*
Tomasz Brzozowski, *Cracow*
Halina Cichoż-Lach, *Lublin*
Andrzej Dabrowski, *Bialystok*
Hanna Gregorek, *Warsaw*
Marek Hartleb, *Katowice*
Beata Jolanta Jablońska, *Katowice*
Stanislaw J Konturek, *Krakow*
Jan Kulig, *Krakow*
Dariusz M Lebensztejn, *Bialystok*
Julian Swierczynski, *Gdansk*

Portugal

Raquel Almeida, *Porto*
Ana Isabel Lopes, *Lisboa Codex*
Ricardo Marcos, *Porto*
Guida Portela-Gomes, *Estoril*

Romania

Dan L Dumitrascu, *Cluj*
Adrian Saftoiu, *Craiova*
Andrada Seicean, *Cluj-Napoca*

Russia

Vasiliy I Reshetnyak, *Moscow*

Saudi Arabia

Ibrahim A Al Mofleh, *Riyadh*
Abdul-Wahed Meshikhes, *Qatif*
Faisal Sanai, *Riyadh*

Serbia

Tamara M Alempijevic, *Belgrade*
Dusan M Jovanovic, *Sremska Kamenica*
Zoran Krivokapic, *Belgrade*

Singapore

Madhav Bhatia, *Singapore*
Kong Weng Eu, *Singapore*
Brian Kim Poh Goh, *Singapore*
Khek-Yu Ho, *Singapore*
Kok Sun Ho, *Singapore*
Fock Kwong Ming, *Singapore*
London Lucien Ooi, *Singapore*
Nagarajan Perumal, *Singapore*
Francis Seow-Choen, *Singapore*

South Africa

Rosemary Joyce Burnett, *Pretoria*
Michael Kew, *Cape Town*

South Korea

Sang Hoon Ahn, *Seoul*
Sung-Gil Chi, *Seoul*
Myung-Gyu Choi, *Seoul*
Hoon Jai Chun, *Seoul*
Yeun-Jun Chung, *Seoul*
Young-Hwa Chung, *Seoul*
Kim Donghee, *Seoul*
Ki-Baik Hahm, *Incheon*
Sun Pyo Hong, *Geonggi-do*
Seong Gyu Hwang, *Seongnam*
Hong Joo Kim, *Seoul*
Jae J Kim, *Seoul*
Jin-Hong Kim, *Suwon*
Nayoung Kim, *Seongnam-si*
Sang Geon Kim, *Seoul*
Seon Hahn Kim, *Seoul*
Sung Kim, *Seoul*
Won Ho Kim, *Seoul*
Jeong Min Lee, *Seoul*
Kyu Taek Lee, *Seoul*
Sang Kil Lee, *Seoul*
Sang Yeoup Lee, *Gyeongsangnam-do*
Yong Chan Lee, *Seoul*
Eun-Yi Moon, *Seoul*
Hyoung-Chul Oh, *Seoul*
Seung Woon Paik, *Seoul*
Joong-Won Park, *Goyang*
Ji Kon Ryu, *Seoul*
Si Young Song, *Seoul*
Marie Yeo, *Suwon*
Byung Chul Yoo, *Seoul*
Dae-Yeul Yu, *Daejeon*

Spain

Maria-Angeles Aller, *Madrid*
Raul J Andrade, *Málaga*
Luis Aparisi, *Valencia*
Gloria González Aseguinolaza, *Navarra*
Matias A Avila, *Pamplona*

Fernando Azpiroz, *Barcelona*
Ramon Bataller, *Barcelona*
Belén Beltrán, *Valencia*
Adolfo Benages, *Valencia*
Josep M Bordas, *Barcelona*
Lisardo Boscá, *Madrid*
Luis Bujanda, *San Sebastián*
Juli Busquets, *Barcelona*
Matilde Bustos, *Pamplona*
José Julián calvo Andrés, *Salamanca*
Andres Cardenas, *Barcelona*
Antoni Castells, *Barcelona*
Fernando J Corrales, *Pamplona*
J E Domínguez-Muñoz, *Santiago de Compostela*
Juan Carlos Laguna Egea, *Barcelona*
Isabel Fabregat, *Barcelona*
Antoni Farré, *Barcelona*
Vicente Felipo, *Valencia*
Laureano Fernández-Cruz, *Barcelona*
Luis Grande, *Barcelona*
Angel Lanas, *Zaragoza*
Juan-Ramón Larrubia, *Guadalajara*
María IT López, *Jaén*
Juan Macías, *Seville*
Javier Martin, *Granada*
José Manuel Martin-Villa, *Madrid*
Julio Mayol, *Madrid*
Mireia Miquel, *Sabadell*
Albert Parés, *Barcelona*
Jesús M Prieto, *Pamplona*
Pedro L Majano Rodriguez, *Madrid*
Joan Roselló-Catafau, *Barcelona*
Eva Vaquero, *Barcelona*

Sweden

Lars Erik Agréus, *Stockholm*
Mats Andersson, *Stockholm*
Roland Andersson, *Lund*
Mauro D'Amato, *Huddinge*
Evangelos Kalaitzakis, *Gothenburg*
Greger Lindberg, *Stockholm*
Annika Lindblom, *Stockholm*
Sara Lindén, *Göteborg*
Hanns-Ulrich Marschall, *Stockholm*
Pär Erik Myrelid, *Linköping*
Åke Nilsson, *Lund*
Helena Nordenstedt, *Stockholm*
Kjell Öberg, *Uppsala*
Lars A Pahlman, *Uppsala*
Stefan G Pierzynowski, *Lund*
Sara Regnér, *Malmö*
Bobby Tingstedt, *Lund*
Zongli Zheng, *Stockholm*

Switzerland

Pascal Bucher, *Geneva*
Michelangelo Foti, *Geneva*
Jean L Frossard, *Geneva*
Andreas Geier, *Zürich*
Pascal Gervaz, *Geneva*
Gerd A Kullak-Ublick, *Zürich*
Fabrizio Montecucco, *Geneva*
Paul M Schneider, *Zürich*
Felix Stickel, *Berne*
Bruno Stieger, *Zürich*
Inti Zlobec, *Basel*

Trinidad and Tobago

Shivananda Nayak, *Mount Hope*

Turkey

Sinan Akay, *Tekirdag*
Metin Basaranoglu, *Istanbul*
Yusuf Bayraktar, *Ankara*
A Mithat Bozdayi, *Ankara*
Hayrullah Derici, *Balıkesir*
Eren Ersoy, *Ankara*
Mukaddes Esrefoglu, *Malatya*
Can Goen, *Kutahya*
Selin Kapan, *Istanbul*
Aydin Karabacakoglu, *Konya*
Cuneyt Kayaalp, *Malatya*
Kemal Kismet, *Ankara*
Seyfettin Köklü, *Ankara*
Mehmet Refik Mas, *Etlik-Ankara*
Osman C Ozdogan, *Istanbul*
Bülent Salman, *Ankara*
Orhan Sezgin, *Mersin*
Ilker Tasci, *Ankara*
Müge Tecder-Ünal, *Ankara*
Ahmet Tekin, *Mersin*
Mesut Tez, *Ankara*
Ekmel Tezel, *Ankara*
Özlem Yilmaz, *Izmir*

United Arab Emirates

Fikri M Abu-Zidan, *Al-Ain*
Sherif M Karam, *Al-Ain*

United Kingdom

Simon Afford, *Birmingham*
Navneet K Ahluwalia, *Stockport*
Mohamed H Ahmed, *Southampton*
Basil Ammori, *Salford*
Lesley A Anderson, *Belfast*
Chin Wee Ang, *Liverpool*
Yeng S Ang, *Wigan*
Anthony TR Axon, *Leeds*
Kathleen B Bamford, *London*
Jim D Bell, *London*
John Beynon, *Swansea*
Chris Briggs, *Sheffield*
Geoffrey Burnstock, *London*
Alastair D Burt, *Newcastle*
Jeff Butterworth, *Shrewsbury*
Jeremy FL Cobbold, *London*
Jean E Crabtree, *Leeds*
Tatjana Crnogorac-Jurcevic, *London*
William Dickey, *Londonderry*
Sunil Dolwani, *Cardiff*
Emad M El-Omar, *Aberdeen*
A M El-Tawil, *Birmingham*
Charles B Ferguson, *Belfast*
Andrew Fowell, *Southampton*
Piers Gatenby, *London*
Daniel R Gaya, *Edinburgh*
Anil George, *London*
Rob Glynne-Jones, *Northwood*
Jason CB Goh, *Birmingham*
Gianpiero Gravante, *Leicester*
Brian Green, *Belfast*
William Greenhalf, *Liverpool*
Indra N Guha, *Nottingham*
Stefan G Hübscher, *Birmingham*
Robin Hughes, *London*
Pali Hungin, *Stockton*
Nawfal Hussein, *Nottingham*
Clement W Imrie, *Glasgow*
Janusz AZ Jankowski, *Oxford*
Sharad Karandikar, *Birmingham*
Peter Karayiannis, *London*
Shahid A Khan, *London*
Patricia F Lalor, *Birmingham*
John S Leeds, *Sheffield*
Ian Lindsey, *Oxford*
Hong-Xiang Liu, *Cambridge*
Dileep N Lobo, *Nottingham*
Graham MacKay, *Glasgow*
Mark Edward McAlindon, *Sheffield*
Anne McCune, *Bristol*
Donald Campbell McMillan, *Glasgow*
Giorgina Mieli-Vergani, *London*
Jamie Murphy, *London*
Guy Fairbairn Nash, *Poole*
James Neuberger, *Birmingham*
Patrick O'Dwyer, *Glasgow*
Christos Paraskeva, *Bristol*
Richard Parker, *North Staffordshire*
Thamara Perera, *Birmingham*
Kondragunta Rajendra Prasad, *Leeds*
D Mark Pritchard, *Liverpool*
Alberto Quaglia, *London*
Akhilesh B Reddy, *Cambridge*
Kevin Robertson, *Glasgow*
Sanchoy Sarkar, *Liverpool*
John B Schofield, *Kent*
Marco Senzolo, *Padova*
Venkatesh Shanmugam, *Derby*
Paul Sharp, *London*
Chew Thean Soon, *Manchester*
Aravind Suppiah, *East Yorkshire*
Noriko Suzuki, *Middlesex*
Simon D Taylor-Robinson, *London*
Frank I Tovey, *London*
A McCulloch Veitch, *Wolverhampton*
Vamsi R Velchuru, *Lowestoft*
Sumita Verma, *Brighton*
Catherine Walter, *Cheltenham*
Julian RF Walters, *London*
Roger Williams, *London*

United States

Kareem M Abu-Elmagd, *Pittsburgh*
Sami R Achem, *Florida*
Golo Ahlenstiel, *Bethesda*
Bhupinder S Anand, *Houston*
M Ananthanarayanan, *New York*
Balamurugan N Appakalal, *Minneapolis*
Dimitrios V Avgerinos, *New York*
Shashi Bala, *Worcester*
Anthony J Bauer, *Pittsburgh*
Kevin E Behrns, *Gainesville*
Roberto Bergamaschi, *New York*
Henry J Binder, *New Haven*
Edmund J Bini, *New York*
Wojciech Blonski, *Philadelphia*
Mark Bloomston, *Columbus*
Edward L Bradley III, *Sarasota*
Carla W Brady, *Durham*

David A Brenner, *San Diego*
Adeel A Butt, *Pittsburgh*
Shi-Ying Cai, *New Haven*
Justin MM Cates, *Nashville*
Eugene P Ceppa, *Durham*
Jianyuan Chai, *Long Beach*
Ronald S Chamberlain, *Livingston*
Fei Chen, *Morgantown*
Xian-Ming Chen, *Omaha*
Ramsey Chi-man Cheung, *Palo Alto*
Denesh Chitkara, *East Brunswick*
Clifford S Cho, *Madison*
Parimal Chowdhury, *Arkansas*
John David Christein, *Birmingham*
Thomas Clancy, *Boston*
Ana J Coito, *Los Angeles*
Ricardo Alberto Cruciani, *New York*
Joseph J Cullen, *Iowa City*
Mark J Czaja, *New York*
Mariana D Dabeva, *Bronx*
Jessica A Davila, *Houston*
Conor P Delaney, *Cleveland*
Laurie DeLeve, *Los Angeles*
Anthony J Demetris, *Pittsburgh*
Sharon DeMorrow, *Temple*
Bijan Eghtesad, *Cleveland*
Yoram Elitsur, *Huntington*
Mohamad A Eloubeidi, *Alabama*
Wael El-Rifai, *Nashville*
Sukru H Emre, *New Haven*
Giamila Fantuzzi, *Chicago*
Ashkan Farhadi, *Irvine*
Ronnie Fass, *Tucson*
Martín E Fernández-Zapico, *Rochester*
Alessandro Fichera, *Chicago*
Josef E Fischer, *Boston*
Piero Marco Fisichella, *Maywood*
Fritz Francois, *New York*
Glenn T Furuta, *Aurora*
T Clark Gamblin, *Pittsburgh*
Henning Gerke, *Iowa City*
Jean-Francois Geschwind, *Baltimore*
R Mark Ghobrial, *Texas*
John F Gibbs, *Buffalo*
Shannon S Glaser, *Temple*
Ajay Goel, *Dallas*
Jon C Gould, *Madison*
Eileen F Grady, *San Francisco*
James H Grendell, *New York*
John R Grider, *Richmond*
Anna S Gukovskaya, *Los Angeles*
Chakshu Gupta, *St. Joseph*
Grigoriy E Gurvits, *New York*
Hai-Yong Han, *Phoenix*
Yuan-Ping Han, *Los Angeles*
Imran Hassan, *Springfield*
Charles P Heise, *Madison*
Lisa J Herrinton, *Oakland*
Oscar Joe Hines, *Los Angeles*
Samuel B Ho, *San Diego*
Steven Hochwald, *Gainesville*
Richard Hu, *Los Angeles*
Eric S Hungness, *Chicago*
Jamal A Ibdah, *Columbia*
Atif Iqbal, *Omaha*
Hartmut Jaeschke, *Tucson*
Donald M Jensen, *Chicago*
Robert Jensen, *Bethesda*
Leonard R Johnson, *Memphis*
Andreas M Kaiser, *Los Angeles*
JingXuan Kang, *Charlestown*
John Y Kao, *Michigan*
Randeep Singh Kashyap, *New York*
Rashmi Kaul, *Tulsa*
Jonathan D Kaunitz, *Los Angeles*
Stephen M Kavic, *Baltimore*
Ali Keshavarzian, *Chicago*
Amir Maqbul Khan, *Marshall*
Kusum K Kharbanda, *Omaha*
Chang Kim, *West Lafayette*
Dean Y Kim, *Detroit*
Miran Kim, *Providence*
Burton I Korelitz, *New York*
Josh Korzenik, *Boston*
Richard A Kozarek, *Seattle*
Alyssa M Krasinskas, *Pittsburgh*
Shiu-Ming Kuo, *Buffalo*
Michelle Lai, *Boston*
Michael Leitman, *New York*
Dong-Hui Li, *Houston*
Ming Li, *New Orleans*
Zhiping Li, *Baltimore*
Gary R Lichtenstein, *Philadelphia*
Chen Liu, *Gainesville*
Zhang-Xu Liu, *Los Angeles*
Craig D Logsdon, *Houston*
Kaye M Reid Lombardo, *Rochester*
Michael R Lucey, *Madison*
Kirk Ludwig, *Wisconsin*
James D Luketich, *Pittsburgh*
Patrick M Lynch, *Houston*
John S Macdonald, *New York*
Willis C Maddrey, *Dallas*
Mercedes Susan Mandell, *Aurora*
Christopher Mantyh, *Durham*
Wendy M Mars, *Pittsburgh*
John Marshall, *Columbia*
Robert CG Martin, *Louisville*
Laura E Matarese, *Pittsburgh*
Craig J McClain, *Louisville*
Lynne V McFarland, *Washington*
David J McGee, *Shreveport*
Valentina Medici, *Sacramento*
Stephan Menne, *New York*
Didier Merlin, *Atlanta*
George Michalopoulos, *Pittsburgh*
James M Millis, *Chicago*
Pramod K Mistry, *New Haven*
Emiko Mizoguchi, *Boston*
Huanbiao Mo, *Denton*
Robert C Moesinger, *Ogden*
Smruti R Mohanty, *Chicago*
John Morton, *Stanford*
Peter L Moses, *Burlington*
Sandeep Mukherjee, *Omaha*
Million Mulugeta, *Los Angeles*
Michel M Murr, *Tampa*
Pete Muscarella, *Columbus*
Ece A Mutlu, *Chicago*
Masaki Nagaya, *Boston*
Laura E Nagy, *Cleveland*
Aejaz Nasir, *Tampa*
Udayakumar Navaneethan, *Cincinnati*
Stephen JD O'Keefe, *Pittsburgh*
Robert D Odze, *Boston*
Giuseppe Orlando, *Winston Salem*
Pal Pacher, *Rockville*
Georgios Papachristou, *Pittsburgh*
Jong Park, *Tampa*
William R Parker, *Durham*
Mansour A Parsi, *Cleveland*
Marco Giuseppe Patti, *Chicago*
Zhiheng Pei, *New York*
CS Pitchumoni, *New Brunswiuc*
Parviz M Pour, *Omaha*
Xiaofa Qin, *Newark*
Florencia Georgina Que, *Rochester*
Massimo Raimondo, *Jacksonville*
Raymund R Razonable, *Minnesota*
Kevin Michael Reavis, *Orange*
Robert V Rege, *Dallas*
Douglas K Rex, *Indianapolis*
Victor E Reyes, *Galveston*
Basil Rigas, *New York*
Richard A Rippe, *Chapel Hill*
Alexander S Rosemurgy, *Tampa*
Philip Rosenthal, *San Francisco*
Raul J Rosenthal, *Weston*
Joel H Rubenstein, *Ann Arbor*
Shawn D Safford, *Norfolk*
Rabih M Salloum, *Rochester*
Bruce E Sands, *Boston*
Tor C Savidge, *Galveston*
Michael L Schilsky, *New Haven*
Beat Schnüriger, *California*
Robert E Schoen, *Pittsburgh*
Matthew James Schuchert, *Pittsburgh*
Ekihiro Seki, *La Jolla*
Le Shen, *Chicago*
Perry Shen, *Winston-Salem*
Stuart Sherman, *Indianapolis*
Mitchell L Shiffman, *Richmond*
Shivendra Shukla, *Columbia*
Bronislaw L Slomiany, *Newark*
Scott Steele, *Fort Lewis*
Branko Stefanovic, *Tallahassee*
Lygia Stewart, *San Francisco*
Luca Stocchi, *Cleveland*
Daniel S Straus, *Riverside*
Robert Todd Striker, *Madison*
Jonathan Strosberg, *Tampa*
Christina Surawicz, *Seattle*
Patricia Sylla, *Boston*
Wing-Kin Syn, *Durham*
Yvette Taché, *Los Angeles*
Kazuaki Takabe, *Richmond*
Kam-Meng Tchou-Wong, *New York*
Klaus Thaler, *Columbia*
Charles Thomas, *Oregon*
Natalie J Torok, *Sacramento*
George Triadafilopoulos, *Stanford*
Chung-Jyi Tsai, *Lexington*
Thérèse Tuohy, *Salt Lake City*
Andrew Ukleja, *Florida*
Santhi Swaroop Vege, *Rochester*
Aaron Vinik, *Norfolk*
Dinesh Vyas, *Washington*
Arnold Wald, *Wisconsin*
Scott A Waldman, *Philadelphia*
Jack R Wands, *Providence*
Jiping Wang, *Boston*
Irving Waxman, *Chicago*
Wilfred M Weinstein, *Los Angeles*
Steven D Wexner, *Weston*
John W Wiley, *Ann Arbor*
Jackie Wood, *Ohio*
Jian Wu, *Sacramento*
Wen Xie, *Pittsburgh*
Guang-Yin Xu, *Galveston*
Fang Yan, *Nashville*
Radha Krishna Yellapu, *New York*
Anthony T Yeung, *Philadelphia*
Zobair M Younossi, *Virginia*
Liqing Yu, *Winston-Salem*
Run Yu, *Los Angeles*
Ruben Zamora, *Pittsburgh*
Michael E Zenilman, *New York*
Mark A Zern, *Sacramento*
Lin Zhang, *Pittsburgh*
Martin D Zielinski, *Rochester*
Michael A Zimmerman, *Colorado*

World Journal of Gastroenterology

Contents

Weekly Volume 17 Number 24 June 28, 2011

EDITORIAL

2879 How to protect liver graft with nitric oxide
Ben Abdennebi H, Zaoualí MA, Alfany-Fernandez I, Tabka D, Roselló-Catafau J

TOPIC HIGHLIGHT

2890 Probiotics in hepatology
Lata J, Jurankova J, Kopacova M, Vitek P

2897 Molecular biology of pancreatic cancer
Zavoral M, Minarikova P, Zavada F, Salek C, Minarik M

ORIGINAL ARTICLE

2909 Genomic imbalances in esophageal carcinoma cell lines involve Wnt pathway genes
Brown J, Bothma H, Veale R, Willem P

2924 Characterization of a novel rat cholangiocarcinoma cell culture model-CGCCA
Yeh CN, Lin KJ, Chen TW, Wu RC, Tsao LC, Chen YT, Weng WH, Chen MF

2933 α-fetoprotein involvement during glucocorticoid-induced precocious maturation in rat colon
Chen M, Sun P, Liu XY, Dong D, Du J, Gu L, Ge YB

BRIEF ARTICLE

2941 Is hyperhomocysteinemia relevant in patients with celiac disease?
Casella G, Bassotti G, Villanacci V, Di Bella C, Pagni F, Corti GL, Sabatino G, Piatti M, Baldini V

2945 Long-term effects of lamivudine treatment in Japanese chronic hepatitis B patients
Murata M, Furusyo N, Unno M, Ogawa E, Toyoda K, Taniai H, Ohnishi H, Hayashi J

2953 Total embolization of the main splenic artery as a supplemental treatment modality for hypersplenism
He XH, Li WT, Peng WJ, Li GD, Wang SP, Xu LC

2958 Antitumor activity of mutant bacterial cytosine deaminase gene for colon cancer
Deng LY, Wang JP, Gui ZF, Shen LZ

Contents

2965 CD133$^+$ gallbladder carcinoma cells exhibit self-renewal ability and tumorigenicity
Shi CJ, Gao J, Wang M, Wang X, Tian R, Zhu F, Shen M, Qin RY

CASE REPORT

2972 Pseudopneumoperitoneum in chronic intestinal pseudo-obstruction: A case report
Camera L, Calabrese M, Sarnelli G, Longobardi M, Rocco A, Cuomo R, Salvatore M

LETTERS TO THE EDITOR

2976 Hepatocellular carcinoma and industrial epidemics
Braillon A, Dubois G

World Journal of Gastroenterology
Volume 17 Number 24 June 28, 2011

Contents

ACKNOWLEDGMENTS I Acknowledgments to reviewers of *World Journal of Gastroenterology*

APPENDIX I Meetings
I-VI Instructions to authors

ABOUT COVER

Brown J, Bothma H, Veale R, Willem P. Genomic imbalances in esophageal carcinoma cell lines involve Wnt pathway genes.
World J Gastroenterol 2011; 17(24): 2909-2923
http://www.wjgnet.com/1007-9327/full/v17/i24/2909.htm

AIM AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

FLYLEAF I-VII Editorial Board

EDITORS FOR THIS ISSUE

Responsible Assistant Editor: *Xiao-Fang Liu*
Responsible Electronic Editor: *Wen-Hua Ma*
Proofing Editor-in-Chief: *Lian-Sheng Ma*
Responsible Science Editor: *Lin Tian*
Proofing Editorial Office Director: *Jian-Xia Cheng*

NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PUBLISHING
Baishideng Publishing Group Co., Limited
Room 1701, 17/F, Henan Building,
No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-3115-8812
Telephone: +852-5804-2046
E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

SUBSCRIPTION
Beijing Baishideng BioMed Scientific Co., Ltd.
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

PUBLICATION DATE
June 28, 2011

ISSN AND EISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

HONORARY EDITORS-IN-CHIEF
James L Boyer, *New Haven*
Ke-Ji Chen, *Beijing*
Martin H Floch, *New Haven*
Geng-Tao Liu, *Beijing*
Emmet B Keeffe, *Palo Alto*
Lein-Ray Mo, *Tainan*
Eamonn M Quigley, *Cork*
Rafiq A Sheikh, *Sacramento*
Nicholas J Talley, *Rochester*
Ming-Lung Yu, *Kaohsiung*

PRESIDENT AND EDITOR-IN-CHIEF
Lian-Sheng Ma, *Beijing*

ACADEMIC EDITOR-IN-CHIEF
Tauseef Ali, *Oklahoma*
Mauro Bortolotti, *Bologna*
Tarkan Karakan, *Ankara*
Weekitt Kittisupamongkol, *Bangkok*
Anastasios Koulaouzidis, *Edinburgh*
Gerd A Kullak-Ublick, *Zürich*
Bo-Rong Pan, *Xi'an*
Sylvia LF Pender, *Southampton*
Max S Petrov, *Auckland*
George Y Wu, *Farmington*

STRATEGY ASSOCIATE EDITORS-IN-CHIEF
Peter Draganov, *Florida*
Hugh J Freeman, *Vancouver*
Maria Concepción Gutiérrez-Ruiz, *México*
Kazuhiro Hanazaki, *Kochi*
Akio Inui, *Kagoshima*
Kalpesh Jani, *Baroda*
Javier S Martin, *Punta del Este*
Natalia A Osna, *Omaha*
Wei Tang, *Tokyo*
Alan BR Thomson, *Edmonton*
Harry HX Xia, *Hanover*

ASSOCIATE EDITORS-IN-CHIEF
You-Yong Lu, *Beijing*
John M Luk, *Pokfulam*
Hiroshi Shimada, *Yokohama*

EDITORIAL OFFICE
Jian-Xia Cheng, Director
World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

COPYRIGHT
© 2011 Baishideng. Articles published by this Open-Access journal are distributed under the terms of the Creative Commons Attribution Non-commercial License, which permits use, distribution, and reproduction in any medium, provided the original work is properly cited, the use is non commercial and is otherwise in compliance with the license.

SPECIAL STATEMENT
All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

ONLINE SUBMISSION
http://www.wjgnet.com/1007-9327office

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2879

World J Gastroenterol 2011 June 28; 17(24): 2879-2889
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2011 Baishideng. All rights reserved.

EDITORIAL

How to protect liver graft with nitric oxide

Hassen Ben Abdennebi, Mohamed Amine Zaoualí, Izabel Alfany-Fernandez, Donia Tabka, Joan Roselló-Catafau

Hassen Ben Abdennebi, Donia Tabka, Laboratory of Human Physiology, Faculty of Pharmacy, University of Monastir, 5000, Monastir, Tunisia

Mohamed Amine Zaoualí, Izabel Alfany-Fernandez, Joan Roselló-Catafau, Unit of Experimental Hepatic Ischemia-Reperfusion, Institut of d'Investigacions Biomèdiques de Barcelona, Consejo Superior de Investigaciones Científicas, Barcelona, 08036, Spain

Mohamed Amine Zaoualí, Joan Roselló-Catafau, Unitat de Transplantament de Fetge i Viabilitat de l'Empelt, Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona, 08036, Spain

Author contributions: Ben Abdennebi H, Zaoualí MA, Alfany-Fernandez I, Tabka D and Roselló-Catafau J participated in the writing of the review.

Supported by The Ministerio de Sanidad y Consumo (PI081988), Ciber-ehd., Instituto Carlos III and Ministerio de Asuntos Exteriores y de Cooperación/AECI (A02987/09 and A/031197/10). Zaoualí MA is a fellowship-holder from the Catalan Transplantation Society

Correspondence to: Dr. Joan Roselló-Catafau, Unit of Experimental Hepatic Ischemia-Reperfusion, Institut of d'Investigacions Biomèdiques de Barcelona, Consejo Superior de Investigaciones Científicas, C/ Rosselló, Barcelona, 08036, Spain. jrcbam@iibb.csic.es

Telephone: +34-93-3638333 **Fax:** +34-93-3638301

Received: November 17, 2010 **Revised:** January 18, 2011
Accepted: January 25, 2011
Published online: June 28, 2011

Abstract

Organ preservation and ischemia reperfusion injury associated with liver transplantation play an important role in the induction of graft injury. One of the earliest events associated with the reperfusion injury is endothelial cell dysfunction. It is generally accepted that endothelial nitric oxide synthase (e-NOS) is cell-protective by mediating vasodilatation, whereas inducible nitric oxide synthase mediates liver graft injury after transplantation. We conducted a critical review of the literature evaluating the potential applications of regulating and promoting e-NOS activity in liver preservation and transplantation, showing the most current evidence to support the concept that enhanced bioavailability of NO derived from e-NOS is detrimental to ameliorate graft liver preservation, as well as preventing subsequent graft reperfusion injury. This review deals mainly with the beneficial effects of promoting "endogenous" pathways for NO generation, *via* e-NOS inducer drugs in cold preservation solution, surgical strategies such as ischemic preconditioning, and alternative "exogenous" pathways that focus on the enrichment of cold storage liquid with NO donors. Finally, we also provide a basic bench-to-bed side summary of the liver physiology and cell signalling mechanisms that account for explaining the e-NOS protective effects in liver preservation and transplantation.

© 2011 Baishideng. All rights reserved.

Key words: Cold ischemia reperfusion injury; Endothelial nitric oxide synthase; Nitric oxide; Liver graft preservation; Ischemic preconditioning; Liver transplantation

Peer reviewer: Fabio Grizzi, PhD, Laboratories of Quantitative Medicine, Istituto Clinico Humanitas IRCCS, Via Manzoni 56, 20089 Rozzano, Milan, Italy

Ben Abdennebi H, Zaoualí MA, Alfany-Fernandez I, Tabka D, Roselló-Catafau J. How to protect liver graft with nitric oxide. *World J Gastroenterol* 2011; 17(24): 2879-2889 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2879.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2879

INTRODUCTION

Ischemia-reperfusion (IR) injury during liver transplantation (LT) is a complex, multi-factorial process in which numerous mediators and a variety of cells interact, leading to tissue damage. It is one of the major cause of both initial poor function and primary non-function of liver allograft, and is responsible for 81% of re-transplantations during the first week after surgery. An intricate network of hepatic and extra-hepatic mechanisms is involved in the genesis of hepatic IR[1-3].

Cold storage and warm reperfusion are unavoidable steps in transplantation and all grafts undergo some degree of IR injury. The cascade of events involves microvasculature (sinusoidal endothelial cells or SEC), Kupffer cells, Ito cells, parenchyma (hepatocytes) and bile ducts. Cold ischemia during organ storage, which is intentionally applied to reduce the metabolic activity of cells in order to preserve the graft before its transplantation, has a substantial effect on graft function. Also, warm ischemia that begins at implantation has an additional negative impact on graft function and outcome. Whatever the type of attack, liver graft damage initiated during the ischemic phases is exacerbated after reperfusion with oxygen and the reintroduction of blood elements.

Although our understanding of the pathophysiology of IR injury is only partial, many response elements indicate that vascular endothelium disruption, the immune response, oxidative mediators, and several cell-death pathways all play important roles. A variety of mediators have been implicated, including reactive oxygen species (ROS) and inflammatory mediators tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1, transforming growth factor-β, interferon-γ and endotheline (ET)-1. Furthermore, complement and chemokines are also released, which leads to leukocyte recruitment and activation. Upregulated adhesion molecules, including intercellular adhesion molecule 1 and E-selectin, enhance endothelial-immune cell interactions. ROS directly injure many cytoskeletal and functional cellular components, causing cell damage. After IR, direct endothelial damage and abnormal vascular tone occurs as a result of an imbalanced sensitivity to mediators of vasoconstriction and vasodilation, such as ET-1/nitric oxide (NO). Endothelial injury also causes cell swelling and narrowing of the vascular lumen, further reducing blood flow. Finally, key regulators of apoptosis, such as caspases, are upregulated resulting in increased cell death.

It is now largely appreciated that IR associated with LT leads to a rapid endothelial dysfunction characterized by a marked decrease in NO production[4-6]. The decrease in NO bioavailability occurs within the first few minutes after reperfusion, and appears to be due to decreased synthesis of NO by NO synthase (NOS), enhanced inactivation of NO by the overproduction of superoxide anion (O_2-), or both. Experimental studies emphasize that it is essential to minimize the deregulation of hepatic microcirculation during LT[7].

NO is a free-radical diatomic gas of low molecular weight with an unpaired electron[8]. It is highly lipophilic, allowing it to permeate quickly across the cell membranes. Its half-life *in vivo* is a few seconds, and it is rapidly converted to stable nitrites (NO_2-) and nitrates (NO_3-)[8]. Endogenous NO is synthesized from the amino acid precursor L-arginine in an oxidation reaction, catalyzed by the NOS enzymes. This complex reaction requires the presence of co-substrates O_2 and NAD(P)H (nicotinamide adenine dinucleotide phosphate reduced), as well as many cofactors such as flavin adenine dinucleotide, flavin mononucleotide and BH4 (tetrahydrobiopterin)[8,9]. There are three isoforms of NOS expressed in the liver in different cells and in different conditions, namely endothelial NOS (e-NOS), neuronal NOS (n-NOS) and inducible NOS (i-NOS). Both e-NOS and n-NOS are constitutively present in liver. The exact role of n-NOS in the pathophysiology of IR injury during LT remains to be established[10]. n-NOS has been observed within neurons innervating the portal tracts by histochemical methods, and several authors believe that this protein is scarcely expressed in the liver[11,12]. In contrast to inducible NOS, constitutive NOS activation requires Ca^{2+}.

Like any important signaling molecule, NO diffuses freely across cell membranes. Under physiological conditions, NO binds to soluble guanylate cyclase inside cells and then induces the production of large quantities of cGMP (guanosine 5'-monophosphate), which then triggers the signal (Figure 1)[13]. *In vivo,* NO is inactivated mainly by superoxide anion, but other pathways could be involved. The first pathway involves the autoxidation of NO to NO_2- and then to NO_3-. Other pathways may be mediated by metal-catalyzed oxidation reactions. The copper-containing protein ceruloplasmin (P-Cu^{2+}) has been shown to rapidly oxidize NO to NO_2- in physiological conditions[14,15]. In addition to the P-Cu^{2+}-mediated reaction, ferrous deoxygenated hemoglobin (Hb-$Fe^{2+}O_2$; oxyhemoglobin) rapidly converts NO to NO_3-.

e-NOS ACTIVITY AND e-NOS-DERIVED NO PRODUCTION IN LT: PATHOPHYSIOLOGICAL ASPECTS

e-NOS is constitutively expressed in venous and arterial endothelial cells and it produces small quantities of NO (at picomolar levels)[16]. It is the main source of NO in endothelial cells under physiologic conditions[9]. e-NOS is also induced in response to specific extracellular stimuli, such as shear stress[17] and metabolic stress (Figure 1)[18]. e-NOS is localized to the caveolae[19,20], which are microdomains of the plasma membrane that have been implicated in a variety of cellular functions, including signal transduction. Caveolin proteins are the major coat proteins of caveolae, and in endothelial cells e-NOS binds to caveolin-1. Caveolin-1 and other peptides from the caveola region directly inhibit e-NOS activity[21,22]. This complex membrane structure is sensitive to the fluid pressure on the membrane.

The e-NOS activation can also be triggered through the signalling pathway involving serine/threonine kinase Akt [or protein kinase B (PKB)], which in turn is stimulated by the phosphoinositide 3-kinase (PI3K)[23,24]. e-NOS is one of the targets of Akt. An important step in this activation is the phosphorylation by Akt of the serine residue in position 1179 (bovine sequence) or serine 1177 (human sequence) of the e-NOS enzyme[25,26]. Therefore, Akt-dependent e-NOS phosphorylation may be an important mechanism in the attenuation of IR injury after

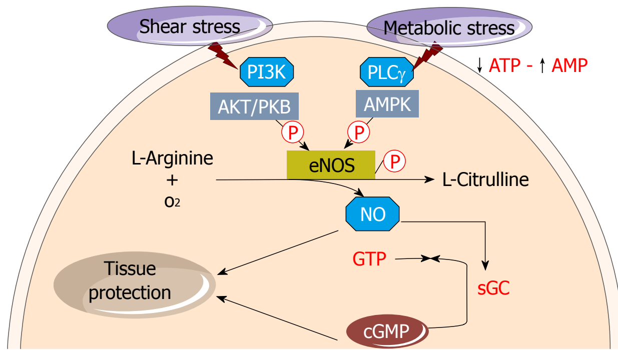


Figure 1 Endothelial nitric oxide synthase-derived nitric oxide synthesis. Shear stress leads to endothelial nitric oxide synthase (e-NOS) phosphorylation and through pathway involving phosphoinositide 3-kinase (PI3K) and Akt. Metabolic stress also phosphorylates e-NOS through the adenosine monophosphate kinase (AMPK) route. The coordination of signalling through these converging pathways allows for e-NOS activation. L-arginine is converted in the endothelium monolayer by the constitutive e-NOS to nitric oxide (NO) and L-citrulline. NO diffuses into both the vessel lumen and the vessel wall, thereby activating soluble guanylate cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP) from guanosine 5'-triphosphate (GTP). NO in concert with cGMP involve tissue protection.

LT. Treatment of liver grafts with adenovirus encoding for myr-Akt improves biochemical and cytoprotective parameters after orthotopic LT in pigs, in comparison to uninfected groups[27].

Recent reports demonstrate that metabolic stress can elicit adenosine monophosphate protein kinase (AMPK), which stimulates phosphorylation of e-NOS protein and increases NO bioavailability in endothelial cells[18,28,29]. The e-NOS-derived NO, in turn, decreases hepatic levels of ET-1, improves hepatic microcirculation and significantly attenuates TNF-α hepatic expression and, remarkably, reduces the activation of caspase-8 and caspase-3 after OLT[30].

In addition to its key role in vascular tone regulation, studies have shown that NO is involved in several other protective routes (Figure 2). Animal studies have shown that early in the reperfusion period, tissue damage appears to be associated with decreased NO availability related to e-NOS down-regulation[27,31]. Similarly, the use of NOS inhibitors leads to IR damage[32]. It has been demonstrated that e-NOS-derived NO inhibits the production and release of several endothelial vasoconstrictor factors, including ET-1[33,34]. NO also interferes with the adhesion and aggregation of platelets, adhesion of leukocytes and monocytes to endothelial cells in vessel walls[35,36]. It also modulates the expression of proinflammatory molecules, such as vascular cell adhesion molecule-1 and monocyte chemoattractant protein 1. Furthermore, NO is emerging as an endogenous inhibitor of TNF-α. NO decreases endothelial permeability and also exerts anti-mitogenic effects in vascular smooth muscle cells by inhibiting their growth and proliferation.

However, other lines of evidence suggest that ROS production after LT reduces e-NOS activation, which becomes uncoupled and perturbs e-NOS-derived NO homeostasis[37] (Figure 3). In parallel, i-NOS is transcriptionally up-regulated in all liver cells, leading to the production of large amounts of NO for persistent periods[37]. Excessive NO generation can be detrimental, because it may alter systemic vascular tone and reactivity, leading to hypotension and circulatory shock[38]. In addition, the generation of peroxynitrite (ONOO-), a potent oxidant formed by reacting NO with O_2-[39], could also cause cell injury through lipid peroxidation, direct inhibition of the mitochondrial respiratory chain[40,41], inhibition of membrane Na^+/K^+ ATPase activity, or oxidative protein modification such as the formation of nitrotyrosine[4,38]. Thus, NO acts as a double-edged sword since it has neither harmful or beneficial effects, depending on its source and the experimental conditions.

The importance of e-NOS for hepatic injury after cold storage/warm reperfusion in transplanted liver grafts has been investigated. The functions of mouse liver grafts retrieved from e-NOS-deficient donors and those from wild-type donors were compared after orthotopic transplantation[31]. e-NOS-deficient liver grafts intensified IR injury, as shown by increased ALT, necrosis and apoptosis, and elevated graft infiltration of monocytes/macrophages. In addition, both flow rate and sinusoidal diameter were diminished after transplantation of e-NOS-deficient grafts. All these alterations are detected from 4 h after LT. In another study, decreased hepatic bioavailability of NO was detected as early as 1 h after reperfusion of human liver transplants[4]. This decline was attributed to a reduction of e-NOS protein levels after reperfusion, rather than to a change in e-NOS mRNA transcription. The concerned mechanism would be a rapid turnover/degradation of the e-NOS enzyme[42]. The extent of the alteration of e-NOS protein expression depended on the duration of preservation, because the loss of e-NOS was exacerbated after 6 h of cold ischemia[43]. The stimulus for the rapid decrease in e-NOS expression is not known.

LT depletes serum arginine due to a massive release of arginase I from cold-injured liver parenchymal cells[44,45]. The depletion of arginine decreases tissue arginine availability, with subsequent down regulation of e-NOS[46,47]. In contrast, enhancement of arginine availability through arginase blockade can protect against hepatic IR injury. The results demonstrate that inhibition of arginase with nor-NOHA can partially reverse the arginine depletion seen in IR injury and improve the histopathological damage following transplantation[48].

MODULATION OF e-NOS-DERIVED NO PRODUCTION FOR LT

The quality of cold preservation is a major detriment of initial graft function and survival. While cold ischemia is considered necessary to slow tissue metabolism, it causes well-documented lesions in the SEC[49]. The SEC is the main target cell of reperfusion injury, at least during the early phase[50]. Endothelial dysfunction leads to NO deficiency, which has been implicated in many disorders.

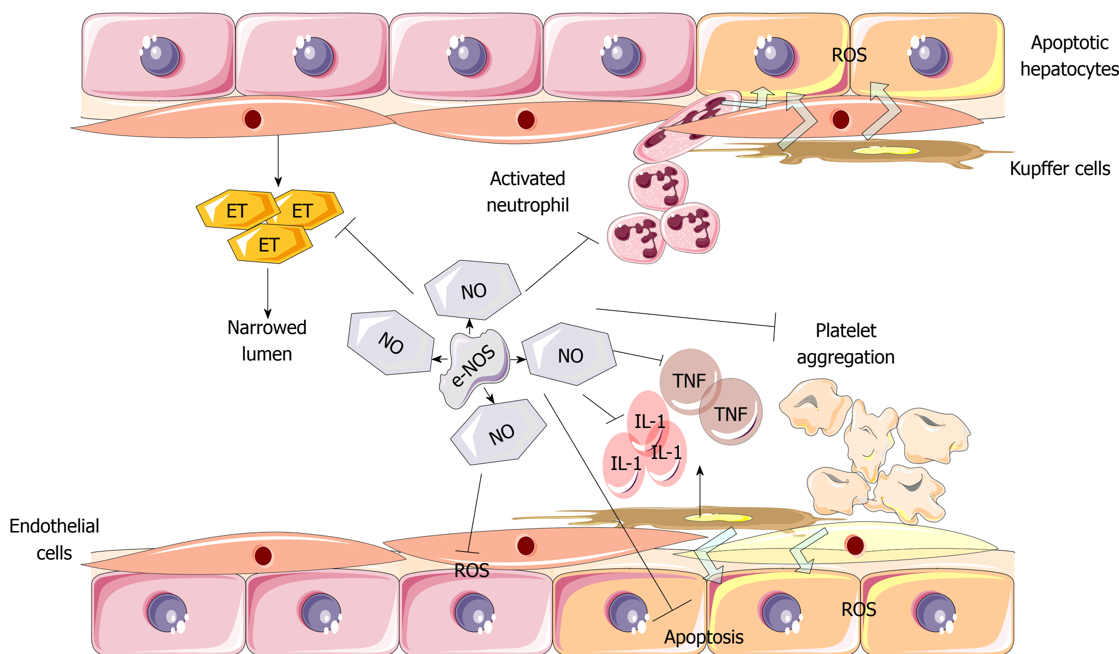


Figure 2 Protective effects of endothelial nitric oxide synthase-derived nitric oxide. The multifactorial consequences are derived from the nitric oxide (NO) generation on oxidative stress (reactive oxygen species), proinflammatory [interleukins (ILs), tumor necrosis factor (TNF)] and vasoconstrictor (endothelins) mediators. e-NOS: Endothelial nitric oxide synthase; ROS: Reactive oxygen species.

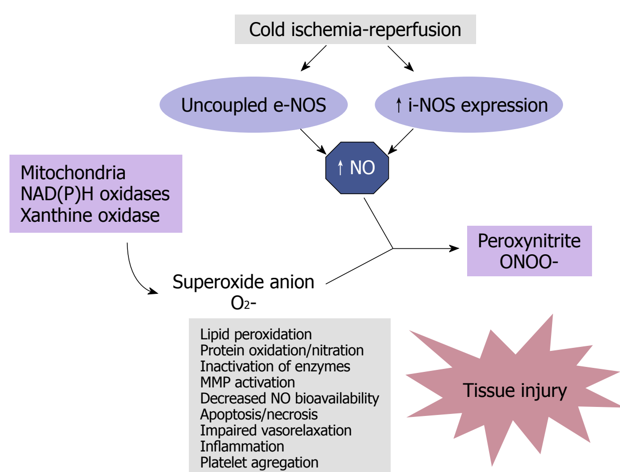


Figure 3 Tissue damage after unbalanced nitric oxide production. Cold ischemia reperfusion involves uncoupled endothelial nitric oxide synthase (e-NOS) and inducible nitric oxide synthase (i-NOS) expression. Large amounts of nitric oxide (NO) are produced under these pathological conditions. NO, in association with increased mitochondrial dysfunction and oxidative stress, reacts with superoxide anion (O_2-), to produce peroxynitrite (ONOO-). Peroxynitrite, in concert with other oxidants, induces tissue damage.

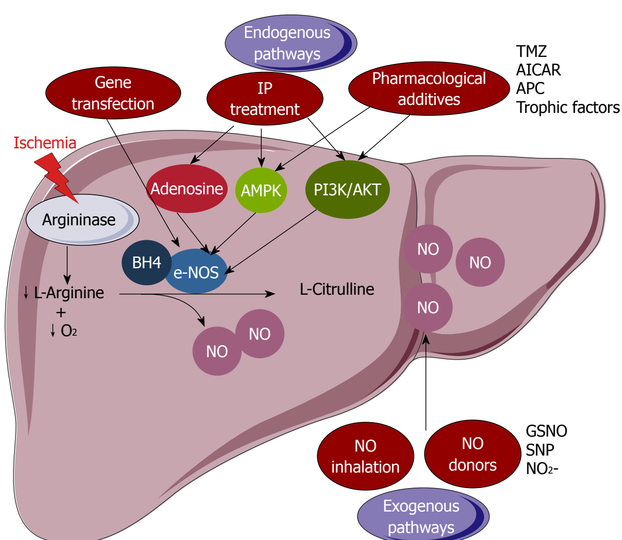


Figure 4 Modulation of the decline in endothelial nitric oxide synthase-derived nitric oxide production. endothelial nitric oxide synthase (e-NOS)-derived nitric oxide (NO) bioavailability can be compromised either by a reduced L-arginine and cofactor (tetrahydrobiopterin, BH4) availability, or by a decreased e-NOS protein activity and level. Two ways seem to be able to overcome these obstacles: the use of exogenous sources of NO (NO donors or NO inhalation) and the induction of the endogenous downstream effectors of e-NOS (pharmacological treatment or ischemic preconditioning) or transfection by an adenovirus containing e-NOS enzyme. SNP: Sodium nitroprusside; GSNO: S-nitroso-L-glutathione; AICAR: 5-amino-4-imidazole carboxamide riboside; TMZ: Trimetazidine; APC: Activated protein C; AMPK: Adenosine monophosphate protein kinase; PI3K: Phosphoinositide 3-kinase.

The morphological changes in these cells interfere with the hepatic microcirculation during reperfusion (adhesion and activation of platelets and leukocytes, and thromboses)[51]. So, improving quality of graft preservation is a means to promote its immediate function, to optimize the allocation of grafts and also to reduce the shortage of organs. Various strategies have been developed to promote immediate recovery of graft function and also to increase access to the marginal donor pool.

Many strategies could be developed to compensate for the decline in NO production during LT. We can distinguish two ways to promote NO production *in vivo*: the endogenous and the exogenous routes (Figure 4). The endogenous way consists of the use of arginine[52]

to compensate for the deficiency of this NO precursor. Other methods consist of directly influencing the activity of the e-NOS enzyme by adding to the preservation solution one of its cofactors (BH4)[53], or one of its inducers, to increase downstream effectors of e-NOS such as Akt and AMPK[54-56]. Transfection of donor liver by an adenovirus containing the e-NOS enzyme could also be envisaged before graft extraction[57,58]. Ischemic preconditioning (IP) could be an endogenous source of NO[59]. The exogenous route requires the use of an exogenous source of NO, which may be given directly by inhalation or indirectly by an NO donor such as sodium nitroprusside (SNP) or S-nitroso-L-glutathione (GSNO)[60-63].

These strategies have shown their effectiveness experimentally, but are not always applicable in human clinical contexts. NO can react with superoxide anions to form ONOO-, a highly reactive oxidant, which can induce apoptosis and cause structural and functional disorders of the graft. This explains why some authors claim that the contribution of NO during cold ischemia may be unnecessarily risky, especially if given at a high dose[64], and the use of NO in humans is controversial at present.

NO GENERATION INDUCED BY THE ENDOGENOUS PATHWAY

Pharmacological e-NOS induction during cold preservation

The potentially protective role of endogenous NO in IR-induced injury to the liver is supported by recently published studies demonstrating enhanced hepatocellular injury in post-ischemic animals that had been rendered deficient in e-NOS[65]. In addition, researchers have demonstrated that over-expression of liver e-NOS protects mice from IR-induced liver injury[27,66-68].

Endogenous e-NOS activation and e-NOS-derived NO might be a promising approach to limiting organ injury. Several pro-survival pathways such as PI3K/Akt and AMPK, which are activated following hepatic injury, are involved in the regulation of e-NOS. Pharmacological up-regulation of these pro-survival kinase cascades may provide an approach to limiting cold ischemic insult. In this regard, 5-amino-4-imidazole carboxamide riboside (AICAR), carvedilol (CVD), trimetazidine (TMZ), activated protein C (APC) and insulin-like growth factor (IGF)-1 could be promising additives in preservation solutions to improve the outcome of liver grafts after cold storage and reperfusion.

Recent studies have shown that the enrichment of University of Wisconsin (UW) with AICAR, an activator of AMPK, ameliorated long-term liver preservation[55]. The protective effect of AICAR on liver injury and function seems to be mediated by enhanced e-NOS activation and NO generation following AMPK phosphorylation, which induces vasodilatation in liver grafts[55]. The relevance of AMPK as an e-NOS upstream regulator was evidenced by the administration of AraA, a phospho-AMPK inhibitor, previous to liver graft preservation, demonstrating that AMPK inhibition reduces e-NOS activation and NO production[55]. In line with these results, Ben Mosbah *et al*[54] demonstrated that the use of CVD, a β- and α-adrenergic blocking drug, when added to UW preservation solution, protected livers through the same mechanism[54]. Livers preserved in this solution show decreased transaminases levels, improved vascular resistance, reduced mitochondrial damage and enhanced ATP levels after reperfusion.

However, the AMPK pathway is not the only e-NOS modulator. Other authors have attempted to improve the histidine-tryptophan-ketoglutarate (HTK) preservation solution by supplementation with an anticoagulant and anti-inflammatory agent, such as APC[30]. They found that the modified HTK preservation solution decreased portal pressure and improved hepatic microcirculation through increased NO hepatic levels *via* up-regulated e-NOS. In addition, the solution attenuated TNF-α expression and markedly reduced the activation of caspase-3 and caspase-8[30]. Although the exact mechanism by which APC activated e-NOS remains unclear, other *in vitro* reports postulated that APC activated e-NOS *via* phosphatidylinositol 3-kinase-dependent phosphorylation, followed by activation of PKB[69].

Recently, Institut Georges Lopez-1 (IGL-1) solution has been proposed as an effective alternative to UW liquid in clinical kidney transplantation, and in experimental orthotopic LT models[70,71]. In addition, we recently demonstrated that IGL-1 is more suitable than UW solution for fatty liver preservation and that the benefits from IGL-1 were linked to an increment of NO synthesis through e-NOS activation which, in turn, reduced oxidative stress and liver injury[72].

TMZ, which has been used as an anti-ischemic drug in the heart for over 35 years, reduced liver injury and improved liver regeneration and survival rate in an experimental model of partial hepatectomy under hepatic blood inflow occlusion[56]. Studies examining the underlying protective mechanisms of TMZ as an additive to UW solution for liver preservation suggest that AMPK up-regulation is the mechanism by which TMZ activates e-NOS and exerts its cytoprotective effect[55]. Another study demonstrated that TMZ attenuated myocardial IR injury *via* PI-3K/AKT kinase pathway activation[73]. We recently found that the addition of TMZ to IGL-1 solution has a synergistic effect on e-NOS-derived NO generation that favours HIF-1α accumulation during normothermic reperfusion[32]. Preserved HIF-1α levels contribute to the increase in the over-expression of cytoprotective proteins such as HO-1 in fatty liver grafts.

It has been established that IGF-1 up-regulates e-NOS activity by interacting with a tyrosine kinase membrane receptor which activates the AKT signalling pathway[74,75]. Furthermore, trophic factors, including IGF-1, have been added to UW solution in an attempt to improve the survival of pig orthotopic liver allografts after cold storage[76]. To this end, we explored the effects of the addition of IGF-1 to IGL-1 solution on fatty liver preservation during cold IRI. We examined the mechanisms

responsible for such effects, including AKT phosphorylation and NO generation. We have demonstrated that the beneficial action of IGF-1 as an additive to IGL-1 is mediated by AKT activation and NO generation, with concomitant prevention of pro-inflammatory cytokines, such as TNF-α[77].

Surgical e-NOS induction by IP

IP is a technique described firstly in the heart by Murry *et al*[78] in 1986, which consists of the application of short and repetitive periods of I/R before a sustained one. The protective effect of IP is not specific to the myocardial muscle, since it is observed in other organs such as skeletal muscle[79], brain[80], intestines[81], lungs[82], kidneys[83] and liver[34]. In any case, the protection induced by IP against IR injury seems to be specific to each organ and animal species, depending on the number of IR cycles applied before the sustained IR. For example, 3-4 cycles are needed for the protection of myocardium[78], whereas in the liver just one cycle of 10 min of ischemia and 10 min of reperfusion is sufficient for maximal protection[59].

In the heart, IP offers an initial protection of 2-3 h after reperfusion, and a remote protection after 12-24 h that lasts for 2 to 3 d. A similar pattern was observed in the liver, although remote protection is not yet well established. Moreover, a differential IP protection was observed in the liver, also depending on the animal species.

Molecular mechanisms responsible for hepatoprotection

The molecular basis of IP is a sequence of episodes triggered by a rapid signal, which leads to an intracellular message and to the amplification of the effector mechanisms of protection[84]. The benefits of IP are caused by the release of several inflammatory mediators such as adenosine and NO, which is followed by the activation of multiple cellular signals. NO is generated by the adenosine released (activation of adenosine A2 receptors), which in turn activates the endothelial constitutive form of the e-NOS enzyme[34,85], a few minutes after IP. The window of liver protection induced by IP is defined by two factors: (1) the concentration of adenosine must be high enough to induce NO; and (2) the concentration of xanthine must be low enough to avoid its prejudicial effects. It is well established that a high concentration of xanthine would support significant increases in superoxide anion. This would react with NO to generate peroxynitrite[86], which would cancel the beneficial effects of IP. Vasodilator effects of NO release improved liver oxygenation and microcirculation[87], and also inhibited the generation of ET, powerful vasoconstrictors generated during liver reperfusion[34].

In addition, IP preserves energy metabolism during sustained ischemia[88,89]. This is confirmed by the maintenance of ATP levels, as well as the depletion of lactate accumulated during the ischemic period. This beneficial effect is mediated by the increase of AMPK, whose activation can be mediated by NO[89].

The activation of the G protein-bound A2 receptor by adenosine stimulates the activity of many intracellular kinases, such as protein-kinase C (PKC) and p38 MAPK[90,91]. In addiction, recent studies implicate PKC in some of the beneficial effects of IP in liver. They show that PKC activation depends on the phosphorylation of different effector molecules, such as the tyrosine kinases[92] and MAPK (including p38 and MAPK[93]), and increases the tolerance of hepatocytes and endothelial cells to the ischemic insult.

Many transcription factors are involved in PKC activation, such as nuclear factor (NF)-κB, which is responsible for the protective effects of PI[94,95]. These transcription factors modulate the expression of particular genes, resulting in the synthesis of proteins such as heat shock proteins (HSP), which are understood to be effectors for the benefits of IP[96]. In the liver, IP is associated with the synthesis of many inducible forms of HSP: HSP70, HSP2 and HSP73 and heme oxygenase (HO-1/HSP32). The induction of HSP depletes the binding between pro-inflammatory transcriptional factors and improves the oxidant capacity of the cells[96-98]. Both effects could contribute to the decrease in TNF-α and to the attenuation of the inflammatory response of preconditioned livers[99,100]. It was also suggested that IP could reduce the transcription of genes, such as *c-fos* and *c-jun*, implicated in the development of the hepatic IRI, and that NFκB activation could induce the activation of signal translator and transcription activator 3, implicated in hepatoprotection and cell proliferation[89,94,96,101,102].

The beneficial role of NFκB in IP is controversial. Whereas Funaki *et al*[103] demonstrate that the protective role of IP is associated with the inhibition of NFκB activation, other authors suggest the opposite, showing that these effects are due to NFκB activation[94,95]. These differences could be attributed to differences in the models used. Besides these cellular signalization pathways, recent studies show that IP could induce the release of small quantities of ROSs[104] and TNF-α, contributing to the protective mechanisms.

Applications to LT

Several studies in animal models have demonstrated the usefulness of IP, but its application to clinical transplantation needs to be clarified. The first clinical application was carried out by Koneru *et al*[105], who used IP in deceased donor LT. They showed that deceased donor liver tolerated 5 min of hilar clamping, but IP did not decrease graft injury. More recently, Azoulay *et al*[106] demonstrated that the effects of 10 min of IP of the liver graft in the donor are associated with better tolerance to ischemia, as well as a worsened early liver function. These studies are consistent with those by Jassem *et al*[107], who demonstrated the protection of cadaver donor livers subjected to IP prior to retrieval by clamping of the hepatic pedicle for 10 min at 24 h after transplantation. This was evidenced by a significant decrease in transaminase levels, and a concomitant reduction of the non-specific inflammatory response.

Similar results were obtained by Cescon *et al*[108], in a prospective randomized study on cadaver donors, by the use of 10 min of IP followed by 15 min of reperfusion. These authors also demonstrated a significant reduction in AST, ALT and i-NOS expression levels after transplantation.

Taking all this into account, new research work is needed to establish the "effective protection" window in human LT and confirm the usefulness of IP in clinical transplantation, including the marginal graft donors, which at present are discarded for clinical transplantation purposes.

NO GENERATION INDUCED BY THE "EXOGENOUS PATHWAY" DURING COLD PRESERVATION

Some considerations

Many teams have sought to improve the performance of storage solutions by supplementation with cytoprotective agents. The bulk of the work on these specific modifications was performed on cellular models in rodents. Unfortunately, the benefits observed were not always confirmed in humans and these changes have often led to inconclusive results in clinical practice. Several NO donors have been tested experimentally for their protective effects on cold IR injury. However, due to the numerous possible reactions and related biological consequences, inappropriate NO levels can cause a series of disease states. On the other hand, insufficient NO production also has serious medical consequences. NO donor therapy should aim to achieve the production of the correct quantity of NO in the correct place for the correct length of time. The exogenous NO should acts primarily as a local mediator to respond to specific stimuli, and then it should simply dissipate through diffusion and oxidation to NO_2- and NO_3-, without the need for complex catabolism. The chemical versatility of NO has led to the synthesis of a wide range of NO donors, each with different modes and rates of NO release. NO donors are pharmacologically active substances that spontaneously release NO (direct donors) or are metabolized to NO (donors requiring metabolism)[109].

The selection of one among these NO donors for therapeutic uses is not easy, since it must meet certain requirements. The compound must be highly soluble in aqueous solutions and diffuse easily into cells, where it produces NO. It must be a proven NO donor, remain in a subtoxic range, have a prolonged half-life and mimic the effect of the endogenous NO. Several authors have amply evaluated the consequences of the use of NO donors in liver under warm ischemia conditions, but little work has been done on the effects on cold ischemia and graft preservation. In our opinion the use of such donors as additive to preservation solution is limited by their short half-life.

Kuroki *et al*[110] studied the effects of SNP on liver warm IR injury. They reported improvement of liver microcirculation and hepatocyte injury in the early period of reperfusion. In another study supporting this finding, SNP infusion after a short period of liver ischemia also decreased liver IR injury[111]. Interestingly, deleterious effects observed during cold storage conditions (like vacuolated hepatocytes, increase in intrahepatic resistance and diminution of bile production) were significantly removed after addition of 500 mmol/L SNP to the UW preservation solution[60]. The authors assumed that beneficial effects of SNP were mediated by NO release[63].

In addition, GSNO has been evaluated in the liver. GSNO may serve as an endogenous long-lived adduct or carrier of NO[112]. GSNO possesses significant antiplatelet action at doses that cause mild hemodynamic effects[113]. Thus, one possible mechanism by which the NO-donor can protect the graft against IR-induced damage could be based on its ability to block platelet aggregation, thus avoiding the intravascular coagulation that can occur during reperfusion. Quintana *et al*[60,61] evaluated the benefit of the addition in UW solution of GSNO as a NO donor. After assaying four GSNO concentrations (50, 100, 250 and 500 mmol/L), they reported an improvement in the properties of UW solution when it contained 100 mmol/L GSNO. It preserved the morphology of hepatocytes and endothelial cells and prevented the alteration of the hemodynamics and function of livers after cold preservation/reperfusion.

Some authors conclude that nitrite (NO_2-) therapy might prove beneficial in protecting organ function and integrity during periods of IR such as those encountered in organ transplantation[114-116]. Under physiological conditions of pH and oxygen pressure, NO_2- has been shown to be a non-active metabolic end product of NO oxidation with limited intrinsic biological activity[117]. However, under ischemic conditions, when e-NOS activity is strongly decreased as a result of its essential dependence on oxygen and the depletion on arginine, an increasing body of evidence indicates alternative NO production by NOS-independent routes. For instance, nitrite may be reduced back to NO by the nitrite reductase action of deoxygenated hemoglobin[118], acidic disproportionation[119], or xanthine oxidoreductase (XOR)[120,121]. In summary, the use of NO_2- in the field of cold preservation could be considered for the following reasons: (1) it is a highly stable substance with no potentially toxic effect; (2) it selectively releases NO under conditions that subsist in stored tissue, namely ischemia, hypoxia, or low pH; (3) it increases XOR activity, which may compensate for compromised constitutive e-NOS activity in terms of NO production, during hypoxia and acidosis; and (4) sodium nitrite is an FDA-approved compound. However, the use of nitrite in preservation solutions that contain allopurinol (such as UW or IGL-1 solutions) would be ineffective, since this latter inhibits XOR.

NEW DIRECTIONS FOR THE FUTURE

Along these lines we have evidenced the relevance of e-NOS system as a useful tool for liver preservation

against cold ischemia reperfusion injury. However, new potential strategies should be established in the future to increase e-NOS activity and modulate the NO availability, as well as to define the appropriate "therapeutic window" to provide the most suitable graft protection during cold storage and further during LT. A better knowledge of understanding the molecular pathways involved may lead to more efficient protective strategies to prevent early cold reperfusion injury during transplantation based on multifactorial activation of the endogenous cytoprotective e-NOS existing in preserved liver grafts.

ACKNOWLEDGMENTS

We are grateful to Robin Rycroft at the Language Advisory Service of the University of Barcelona for revising the English text. Also Catalan Society of Transplantation was acknowledged by the fellow to Mohamed Amine Zaoualí.

REFERENCES

1 **Clavien PA**, Harvey PR, Strasberg SM. Preservation and reperfusion injuries in liver allografts. An overview and synthesis of current studies. *Transplantation* 1992; **53**: 957-978
2 **Casillas-Ramírez A**, Mosbah IB, Ramalho F, Roselló-Catafau J, Peralta C. Past and future approaches to ischemia-reperfusion lesion associated with liver transplantation. *Life Sci* 2006; **79**: 1881-1894
3 **Jaeschke H**. Preservation injury: mechanisms, prevention and consequences. *J Hepatol* 1996; **25**: 774-780
4 **Varadarajan R**, Golden-Mason L, Young L, McLoughlin P, Nolan N, McEntee G, Traynor O, Geoghegan J, Hegarty JE, O'Farrelly C. Nitric oxide in early ischaemia reperfusion injury during human orthotopic liver transplantation. *Transplantation* 2004; **78**: 250-256
5 **Tsuchiya K**, Kanematsu Y, Yoshizumi M, Ohnishi H, Kirima K, Izawa Y, Shikishima M, Ishida T, Kondo S, Kagami S, Takiguchi Y, Tamaki T. Nitrite is an alternative source of NO in vivo. *Am J Physiol Heart Circ Physiol* 2005; **288**: H2163-H2170
6 **Kume M**, Banafsche R, Yamamoto Y, Yamaoka Y, Nobiling R, Gebhard MM, Klar E. Dynamic changes of post-ischemic hepatic microcirculation improved by a pre-treatment of phosphodiesterase-3 inhibitor, milrinone. *J Surg Res* 2006; **136**: 209-218
7 **Rubbo H**, Darley-Usmar V, Freeman BA. Nitric oxide regulation of tissue free radical injury. *Chem Res Toxicol* 1996; **9**: 809-820
8 **Alderton WK**, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem J* 2001; **357**: 593-615
9 **Andrew PJ**, Mayer B. Enzymatic function of nitric oxide synthases. *Cardiovasc Res* 1999; **43**: 521-531
10 **Shah V**, Kamath PS. Nitric oxide in liver transplantation: pathobiology and clinical implications. *Liver Transpl* 2003; **9**: 1-11
11 **Esteban FJ**, Pedrosa JA, Jiménez A, Fernández AP, Bentura ML, Martínez-Murillo R, Rodrigo J, Peinado MA. Distribution of neuronal nitric oxide synthase in the rat liver. *Neurosci Lett* 1997; **226**: 99-102
12 **Park CS**, Krishna G, Ahn MS, Kang JH, Chung WG, Kim DJ, Hwang HK, Lee JN, Paik SG, Cha YN. Differential and constitutive expression of neuronal, inducible, and endothelial nitric oxide synthase mRNAs and proteins in pathologically normal human tissues. *Nitric Oxide* 2000; **4**: 459-471
13 **Vollmar B**, Menger MD. The hepatic microcirculation: mechanistic contributions and therapeutic targets in liver injury and repair. *Physiol Rev* 2009; **89**: 1269-1339
14 **Lundberg JO**, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov* 2008; **7**: 156-167
15 **Lundberg JO**, Weitzberg E. NO-synthase independent NO generation in mammals. *Biochem Biophys Res Commun* 2010; **396**: 39-45
16 **Domenico R**. Pharmacology of nitric oxide: molecular mechanisms and therapeutic strategies. *Curr Pharm Des* 2004; **10**: 1667-1676
17 **Kolluru GK**, Sinha S, Majumder S, Muley A, Siamwala JH, Gupta R, Chatterjee S. Shear stress promotes nitric oxide production in endothelial cells by sub-cellular delocalization of eNOS: A basis for shear stress mediated angiogenesis. *Nitric Oxide* 2010; **22**: 304-315
18 **Bouma HR**, Ketelaar ME, Yard BA, Ploeg RJ, Henning RH. AMP-activated protein kinase as a target for preconditioning in transplantation medicine. *Transplantation* 2010; **90**: 353-358
19 **García-Cardeña G**, Oh P, Liu J, Schnitzer JE, Sessa WC. Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling. *Proc Natl Acad Sci USA* 1996; **93**: 6448-6453
20 **Shaul PW**, Smart EJ, Robinson LJ, German Z, Yuhanna IS, Ying Y, Anderson RG, Michel T. Acylation targets emdothelial nitric-oxide synthase to plasmalemmal caveolae. *J Biol Chem* 1996; **271**: 6518-6522
21 **Ju H**, Zou R, Venema VJ, Venema RC. Direct interaction of endothelial nitric-oxide synthase and caveolin-1 inhibits synthase activity. *J Biol Chem* 1997; **272**: 18522-18525
22 **Michel JB**, Feron O, Sacks D, Michel T. Reciprocal regulation of endothelial nitric-oxide synthase by Ca2+-calmodulin and caveolin. *J Biol Chem* 1997; **272**: 15583-15586
23 **Franke TF**, Kaplan DR, Cantley LC. PI3K: downstream AKTion blocks apoptosis. *Cell* 1997; **88**: 435-437
24 **Downward J**. Mechanisms and consequences of activation of protein kinase B/Akt. *Curr Opin Cell Biol* 1998; **10**: 262-267
25 **Dimmeler S**, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999; **399**: 601-605
26 **Fulton D**, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 1999; **399**: 597-601
27 **Morales-Ruiz M**, Fondevila C, Muñoz-Luque J, Tugues S, Rodríguez-Laiz G, Cejudo-Martín P, Romero JM, Navasa M, Fuster J, Arroyo V, Sessa WC, García-Valdecasas JC, Jiménez W. Gene transduction of an active mutant of akt exerts cytoprotection and reduces graft injury after liver transplantation. *Am J Transplant* 2007; **7**: 769-778
28 **Chen Z**, Peng IC, Sun W, Su MI, Hsu PH, Fu Y, Zhu Y, DeFea K, Pan S, Tsai MD, Shyy JY. AMP-activated protein kinase functionally phosphorylates endothelial nitric oxide synthase Ser633. *Circ Res* 2009; **104**: 496-505
29 **Chen Z**, Peng IC, Cui X, Li YS, Chien S, Shyy JY. Shear stress, SIRT1, and vascular homeostasis. *Proc Natl Acad Sci USA* 2010; **107**: 10268-10273
30 **Kuriyama N**, Isaji S, Hamada T, Kishiwada M, Ohsawa I, Usui M, Sakurai H, Tabata M, Hayashi T, Suzuki K. The cytoprotective effects of addition of activated protein C into preservation solution on small-for-size grafts in rats. *Liver Transpl* 2010; **16**: 1-11
31 **Theruvath TP**, Zhong Z, Currin RT, Ramshesh VK, Lemasters JJ. Endothelial nitric oxide synthase protects transplanted mouse livers against storage/reperfusion injury: Role of vasodilatory and innate immunity pathways. *Transplant Proc* 2006; **38**: 3351-3357
32 **Zaouali MA**, Ben Mosbah I, Boncompagni E, Ben Abdennebi H, Mitjavila MT, Bartrons R, Freitas I, Rimola A, Roselló-Catafau J. Hypoxia inducible factor-1alpha accumulation in

steatotic liver preservation: role of nitric oxide. *World J Gastroenterol* 2010; **16**: 3499-3509

33 **Peralta C**, Rull R, Rimola A, Deulofeu R, Roselló-Catafau J, Gelpí E, Rodés J. Endogenous nitric oxide and exogenous nitric oxide supplementation in hepatic ischemia-reperfusion injury in the rat. *Transplantation* 2001; **71**: 529-536

34 **Peralta C**, Closa D, Hotter G, Gelpí E, Prats N, Roselló-Catafau J. Liver ischemic preconditioning is mediated by the inhibitory action of nitric oxide on endothelin. *Biochem Biophys Res Commun* 1996; **229**: 264-270

35 **Taniai H**, Hines IN, Bharwani S, Maloney RE, Nimura Y, Gao B, Flores SC, McCord JM, Grisham MB, Aw TY. Susceptibility of murine periportal hepatocytes to hypoxia-reoxygenation: role for NO and Kupffer cell-derived oxidants. *Hepatology* 2004; **39**: 1544-1552

36 **Esch JS**, Jurk K, Knoefel WT, Roeder G, Voss H, Tustas RY, Schmelzle M, Krieg A, Eisenberger CF, Topp S, Rogiers X, Fischer L, Aken HV, Kehrel BE. Platelet activation and increased tissue factor expression on monocytes in reperfusion injury following orthotopic liver transplantation. *Platelets* 2010; **21**: 348-359

37 **Hu M**, Wang Z, Rao J, Cao Y, Jiang W, Zhang F, Li X, Wang X. Inhibition of inducible nitric oxide synthase worsens liver damage regardless of lipopolysaccharide treatment in small-for-size liver transplantation. *Transpl Immunol* 2010; **23**: 6-11

38 **Ramalho FS**, Fernandez-Monteiro I, Rosello-Catafau J, Peralta C. Hepatic microcirculatory failure. *Acta Cir Bras* 2006; **21 Suppl 1**: 48-53

39 **Szabó C**. Multiple pathways of peroxynitrite cytotoxicity. *Toxicol Lett* 2003; **140-141**: 105-112

40 **Lacza Z**, Kozlov AV, Pankotai E, Csordás A, Wolf G, Redl H, Kollai M, Szabó C, Busija DW, Horn TF. Mitochondria produce reactive nitrogen species via an arginine-independent pathway. *Free Radic Res* 2006; **40**: 369-378

41 **Lacza Z**, Pankotai E, Csordás A, Gero D, Kiss L, Horváth EM, Kollai M, Busija DW, Szabó C. Mitochondrial NO and reactive nitrogen species production: does mtNOS exist? *Nitric Oxide* 2006; **14**: 162-168

42 **Lang JD**, Teng X, Chumley P, Crawford JH, Isbell TS, Chacko BK, Liu Y, Jhala N, Crowe DR, Smith AB, Cross RC, Frenette L, Kelley EE, Wilhite DW, Hall CR, Page GP, Fallon MB, Bynon JS, Eckhoff DE, Patel RP. Inhaled NO accelerates restoration of liver function in adults following orthotopic liver transplantation. *J Clin Invest* 2007; **117**: 2583-2591

43 **Desrois M**, Caus T, Belles PM, Dalmasso C, Lan C, Cozzone PJ, Bernard M. Nitric oxide pathway after long-term cold storage and reperfusion in a heterotopic rat heart transplantation model. *Transplant Proc* 2005; **37**: 4553-4555

44 **Roth E**, Steininger R, Winkler S, Längle F, Grünberger T, Függer R, Mühlbacher F. L-Arginine deficiency after liver transplantation as an effect of arginase efflux from the graft. Influence on nitric oxide metabolism. *Transplantation* 1994; **57**: 665-669

45 **Yagnik GP**, Takahashi Y, Tsoulfas G, Reid K, Murase N, Geller DA. Blockade of the L-arginine/NO synthase pathway worsens hepatic apoptosis and liver transplant preservation injury. *Hepatology* 2002; **36**: 573-581

46 **Mori M**, Gotoh T. Regulation of nitric oxide production by arginine metabolic enzymes. *Biochem Biophys Res Commun* 2000; **275**: 715-719

47 **Becker T**, Mevius I, de Vries DK, Schaapherder AF, zu Vilsendorf AM, Klempnauer J, Frölich JC, Tsikas D. The L-arginine/NO pathway in end-stage liver disease and during orthotopic liver and kidney transplantation: biological and analytical ramifications. *Nitric Oxide* 2009; **20**: 61-67

48 **Reid KM**, Tsung A, Kaizu T, Jeyabalan G, Ikeda A, Shao L, Wu G, Murase N, Geller DA. Liver I/R injury is improved by the arginase inhibitor, N(omega)-hydroxy-nor-L-arginine (nor-NOHA). *Am J Physiol Gastrointest Liver Physiol* 2007; **292**: G512-G517

49 **Clavien PA**. Sinusoidal endothelial cell injury during hepatic preservation and reperfusion. *Hepatology* 1998; **28**: 281-285

50 **Zhu J**, Wang S, Bie P, Li X, Zhang Y, Xiong Y, Wang H, Ma Z, Li K, Dong J. Apoptosis and regeneration of sinusoidal endothelial cells after extended cold preservation and transplantation of rat liver. *Transplantation* 2007; **84**: 1483-1491

51 **Lemasters JJ**, Thurman RG. Reperfusion injury after liver preservation for transplantation. *Annu Rev Pharmacol Toxicol* 1997; **37**: 327-338

52 **Higashi Y**, Oshima T, Ono N, Hiraga H, Yoshimura M, Watanabe M, Matsuura H, Kambe M, Kajiyama G. Intravenous administration of L-arginine inhibits angiotensin-converting enzyme in humans. *J Clin Endocrinol Metab* 1995; **80**: 2198-2202

53 **Yamashiro S**, Kuniyoshi Y, Arakaki K, Uezu T, Miyagi K, Koja K. Cardioprotective effects of tetrahydrobiopterin in cold heart preservation after cardiac arrest. *Ann Thorac Cardiovasc Surg* 2006; **12**: 95-104

54 **Ben Mosbah I**, Roselló-Catafau J, Alfany-Fernandez I, Rimola A, Parellada PP, Mitjavila MT, Lojek A, Ben Abdennebi H, Boillot O, Rodés J, Peralta C. Addition of carvedilol to University Wisconsin solution improves rat steatotic and nonsteatotic liver preservation. *Liver Transpl* 2010; **16**: 163-171

55 **Ben Mosbah I**, Massip-Salcedo M, Fernández-Monteiro I, Xaus C, Bartrons R, Boillot O, Roselló-Catafau J, Peralta C. Addition of adenosine monophosphate-activated protein kinase activators to University of Wisconsin solution: a way of protecting rat steatotic livers. *Liver Transpl* 2007; **13**: 410-425

56 **Zaouali MA**, Ben Abdennebi H, Padrissa-Altés S, Mahfoudh-Boussaid A, Roselló-Catafau J. Pharmacological strategies against cold ischemia reperfusion injury. *Expert Opin Pharmacother* 2010; **11**: 537-555

57 **Kupatt C**, Hinkel R, von Brühl ML, Pohl T, Horstkotte J, Raake P, El Aouni C, Thein E, Dimmeler S, Feron O, Boekstegers P. Endothelial nitric oxide synthase overexpression provides a functionally relevant angiogenic switch in hibernating pig myocardium. *J Am Coll Cardiol* 2007; **49**: 1575-1584

58 **Kaur S**, Kumar TR, Uruno A, Sugawara A, Jayakumar K, Kartha CC. Genetic engineering with endothelial nitric oxide synthase improves functional properties of endothelial progenitor cells from patients with coronary artery disease: an in vitro study. *Basic Res Cardiol* 2009; **104**: 739-749

59 **Peralta C**, Hotter G, Closa D, Gelpí E, Bulbena O, Roselló-Catafau J. Protective effect of preconditioning on the injury associated to hepatic ischemia-reperfusion in the rat: role of nitric oxide and adenosine. *Hepatology* 1997; **25**: 934-937

60 **Quintana AB**, Rodriguez JV, Scandizzi AL, Guibert EE. The benefit of adding sodium nitroprusside (NPNa) or S-nitrosoglutathion (GSNO) to the University of Wisconsin solution (UW) to prevent morphological alterations during cold preservation/reperfusion of rat livers. *Ann Hepatol* 2003; **2**: 84-91

61 **Quintana A**, Rodriguez JV, Scandizzi A, Guibert EE. Effect of S-nitrosoglutathione (GSNO) added to the University of Wisconsin solution (UW): I) Morphological alteration during cold preservation/reperfusion of rat liver. *Int J Surg Investig* 2001; **2**: 401-411

62 **Miki T**, Subbotin V, Goller AL, Tandin A, Rao AS, Fung JJ, Valdivia LA. Role of UW solution and sodium nitroprusside in reperfusion of liver xenografts from guinea-pig to rat. *Xenotransplantation* 1999; **6**: 117-122

63 **Rodriguez JV**, Guibert EE, Quintana A, Scandizzi A, Almada L. Role of sodium nitroprusside in the improvement of rat liver preservation in University of Wisconsin solution: A study in the isolated perfused liver model. *J Surg Res* 1999; **87**: 201-208

64 **de Perrot M**, Keshavjee S. Lung preservation. *Semin Thorac Cardiovasc Surg* 2004; **16**: 300-308

65 **Hines IN**, Hoffman JM, Scheerens H, Day BJ, Harada H, Pavlick KP, Bharwani S, Wolf R, Gao B, Flores S, McCord JM, Grisham MB. Regulation of postischemic liver injury following different durations of ischemia. *Am J Physiol Gastrointest*

Liver Physiol 2003; **284**: G536-G545
66 **Li J**, Billiar TR. Nitric Oxide. IV. Determinants of nitric oxide protection and toxicity in liver. *Am J Physiol* 1999; **276**: G1069-G1073
67 **Kuhlencordt PJ**, Rosel E, Gerszten RE, Morales-Ruiz M, Dombkowski D, Atkinson WJ, Han F, Preffer F, Rosenzweig A, Sessa WC, Gimbrone MA, Ertl G, Huang PL. Role of endothelial nitric oxide synthase in endothelial activation: insights from eNOS knockout endothelial cells. *Am J Physiol Cell Physiol* 2004; **286**: C1195-C1202
68 **Katsumi H**, Nishikawa M, Yamashita F, Hashida M. Prevention of hepatic ischemia/reperfusion injury by prolonged delivery of nitric oxide to the circulating blood in mice. *Transplantation* 2008; **85**: 264-269
69 **Uchiba M**, Okajima K, Oike Y, Ito Y, Fukudome K, Isobe H, Suda T. Activated protein C induces endothelial cell proliferation by mitogen-activated protein kinase activation in vitro and angiogenesis in vivo. *Circ Res* 2004; **95**: 34-41
70 **Ben Abdennebi H**, Elrassi Z, Scoazec JY, Steghens JP, Ramella-Virieux S, Boillot O. Evaluation of IGL-1 preservation solution using an orthotopic liver transplantation model. *World J Gastroenterol* 2006; **12**: 5326-5330
71 **Badet L**, Ben Abdennebi H, Petruzzo P, McGregor B, Espa M, Hadj-Aissa A, Ramella-Virieux S, Steghens JP, Portoghese F, Martin X. Effect of IGL-1, a new preservation solution, on kidney grafts (a pre-clinical study). *Transpl Int* 2005; **17**: 815-821
72 **Ben Mosbah I**, Roselló-Catafau J, Franco-Gou R, Abdennebi HB, Saidane D, Ramella-Virieux S, Boillot O, Peralta C. Preservation of steatotic livers in IGL-1 solution. *Liver Transpl* 2006; **12**: 1215-1223
73 **Khan M**, Meduru S, Mostafa M, Khan S, Hideg K, Kuppusamy P. Trimetazidine, administered at the onset of reperfusion, ameliorates myocardial dysfunction and injury by activation of p38 mitogen-activated protein kinase and Akt signaling. *J Pharmacol Exp Ther* 2010; **333**: 421-429
74 **Isenovic ER**, Divald A, Milivojevic N, Grgurevic T, Fisher SE, Sowers JR. Interactive effects of insulin-like growth factor-1 and beta-estradiol on endothelial nitric oxide synthase activity in rat aortic endothelial cells. *Metabolism* 2003; **52**: 482-487
75 **Isenovic ER**, Meng Y, Divald A, Milivojevic N, Sowers JR. Role of phosphatidylinositol 3-kinase/Akt pathway in angiotensin II and insulin-like growth factor-1 modulation of nitric oxide synthase in vascular smooth muscle cells. *Endocrine* 2002; **19**: 287-292
76 **Ambiru S**, Uryuhara K, Talpe S, Dehoux JP, Jacobbi L, Murphy CJ, McAnulty JF, Gianello P. Improved survival of orthotopic liver allograft in swine by addition of trophic factors to University of Wisconsin solution. *Transplantation* 2004; **77**: 302-319
77 **Zaouali MA**, Padrissa-Altés S, Ben Mosbah I, Ben Abdennebi H, Boillot O, Rimola A, Saidane-Mosbahi D, Roselló-Catafau J. Insulin like growth factor-1 increases fatty liver preservation in IGL-1 solution. *World J Gastroenterol* 2010; **16**: 5693-5700
78 **Murry CE**, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986; **74**: 1124-1136
79 **Pang CY**, Yang RZ, Zhong A, Xu N, Boyd B, Forrest CR. Acute ischaemic preconditioning protects against skeletal muscle infarction in the pig. *Cardiovasc Res* 1995; **29**: 782-788
80 **Glazier SS**, O'Rourke DM, Graham DI, Welsh FA. Induction of ischemic tolerance following brief focal ischemia in rat brain. *J Cereb Blood Flow Metab* 1994; **14**: 545-553
81 **Hotter G**, Closa D, Prados M, Fernández-Cruz L, Prats N, Gelpí E, Roselló-Catafau J. Intestinal preconditioning is mediated by a transient increase in nitric oxide. *Biochem Biophys Res Commun* 1996; **222**: 27-32
82 **Du ZY**, Hicks M, Winlaw D, Spratt P, Macdonald P. Ischemic preconditioning enhances donor lung preservation in the rat. *J Heart Lung Transplant* 1996; **15**: 1258-1267
83 **Islam CF**, Mathie RT, Dinneen MD, Kiely EA, Peters AM, Grace PA. Ischaemia-reperfusion injury in the rat kidney: the effect of preconditioning. *Br J Urol* 1997; **79**: 842-847
84 **Cutrn JC**, Perrelli MG, Cavalieri B, Peralta C, Rosell Catafau J, Poli G. Microvascular dysfunction induced by reperfusion injury and protective effect of ischemic preconditioning. *Free Radic Biol Med* 2002; **33**: 1200-1208
85 **Koti RS**, Seifalian AM, McBride AG, Yang W, Davidson BR. The relationship of hepatic tissue oxygenation with nitric oxide metabolism in ischemic preconditioning of the liver. *FASEB J* 2002; **16**: 1654-1656
86 **Peralta C**, Closa D, Xaus C, Gelpí E, Roselló-Catafau J, Hotter G. Hepatic preconditioning in rats is defined by a balance of adenosine and xanthine. *Hepatology* 1998; **28**: 768-773
87 **Koti RS**, Yang W, Dashwood MR, Davidson BR, Seifalian AM. Effect of ischemic preconditioning on hepatic microcirculation and function in a rat model of ischemia reperfusion injury. *Liver Transpl* 2002; **8**: 1182-1191
88 **Peralta C**, Bartrons R, Serafin A, Blázquez C, Guzmán M, Prats N, Xaus C, Cutillas B, Gelpí E, Roselló-Catafau J. Adenosine monophosphate-activated protein kinase mediates the protective effects of ischemic preconditioning on hepatic ischemia-reperfusion injury in the rat. *Hepatology* 2001; **34**: 1164-1173
89 **Carrasco-Chaumel E**, Roselló-Catafau J, Bartrons R, Franco-Gou R, Xaus C, Casillas A, Gelpí E, Rodés J, Peralta C. Adenosine monophosphate-activated protein kinase and nitric oxide in rat steatotic liver transplantation. *J Hepatol* 2005; **43**: 997-1006
90 **Carini R**, Grazia De Cesaris M, Splendore R, Domenicotti C, Nitti MP, Pronzato MA, Albano E. Signal pathway responsible for hepatocyte preconditioning by nitric oxide. *Free Radic Biol Med* 2003; **34**: 1047-1055
91 **Ricciardi R**, Meyers WC, Schaffer BK, Kim RD, Shah SA, Wheeler SM, Donohue SE, Sheth KR, Callery MP, Chari RS. Protein kinase C inhibition abrogates hepatic ischemic preconditioning responses. *J Surg Res* 2001; **97**: 144-149
92 **Ricciardi R**, Schaffer BK, Kim RD, Shah SA, Donohue SE, Wheeler SM, Quarfordt SH, Callery MP, Meyers WC, Chari RS. Protective effects of ischemic preconditioning on the cold-preserved liver are tyrosine kinase dependent. *Transplantation* 2001; **72**: 406-412
93 **Carini R**, De Cesaris MG, Splendore R, Vay D, Domenicotti C, Nitti MP, Paola D, Pronzato MA, Albano E. Signal pathway involved in the development of hypoxic preconditioning in rat hepatocytes. *Hepatology* 2001; **33**: 131-139
94 **Teoh N**, Dela Pena A, Farrell G. Hepatic ischemic preconditioning in mice is associated with activation of NF-kappaB, p38 kinase, and cell cycle entry. *Hepatology* 2002; **36**: 94-102
95 **Ricciardi R**, Shah SA, Wheeler SM, Quarfordt SH, Callery MP, Meyers WC, Chari RS. Regulation of NFkappaB in hepatic ischemic preconditioning. *J Am Coll Surg* 2002; **195**: 319-326
96 **Kume M**, Yamamoto Y, Saad S, Gomi T, Kimoto S, Shimabukuro T, Yagi T, Nakagami M, Takada Y, Morimoto T, Yamaoka Y. Ischemic preconditioning of the liver in rats: implications of heat shock protein induction to increase tolerance of ischemia-reperfusion injury. *J Lab Clin Med* 1996; **128**: 251-258
97 **Redaelli CA**, Tian YH, Schaffner T, Ledermann M, Baer HU, Dufour JF. Extended preservation of rat liver graft by induction of heme oxygenase-1. *Hepatology* 2002; **35**: 1082-1092
98 **Bauer M**, Bauer I. Heme oxygenase-1: redox regulation and role in the hepatic response to oxidative stress. *Antioxid Redox Signal* 2002; **4**: 749-758
99 **Arai M**, Thurman RG, Lemasters JJ. Ischemic preconditioning of rat livers against cold storage-reperfusion injury: role of nonparenchymal cells and the phenomenon of heterologous preconditioning. *Liver Transpl* 2001; **7**: 292-299
100 **Yonezawa K**, Yamamoto Y, Yamamoto H, Ishikawa Y, Uchinami H, Taura K, Nakajima A, Yamaoka Y. Suppression

of tumor necrosis factor-alpha production and neutrophil infiltration during ischemia-reperfusion injury of the liver after heat shock preconditioning. *J Hepatol* 2001; **35**: 619-627

101 **Carini R**, Albano E. Recent insights on the mechanisms of liver preconditioning. *Gastroenterology* 2003; **125**: 1480-1491

102 **Ishii S**, Abe T, Saito T, Tsuchiya T, Kanno H, Miyazawa M, Suzuki M, Motoki R, Gotoh M. Effects of preconditioning on ischemia/reperfusion injury of hepatocytes determined by immediate early gene transcription. *J Hepatobiliary Pancreat Surg* 2001; **8**: 461-468

103 **Funaki H**, Shimizu K, Harada S, Tsuyama H, Fushida S, Tani T, Miwa K. Essential role for nuclear factor kappaB in ischemic preconditioning for ischemia-reperfusion injury of the mouse liver. *Transplantation* 2002; **74**: 551-556

104 **Sindram D**, Rüdiger HA, Upadhya AG, Strasberg SM, Clavien PA. Ischemic preconditioning protects against cold ischemic injury through an oxidative stress dependent mechanism. *J Hepatol* 2002; **36**: 78-84

105 **Koneru B**, Fisher A, He Y, Klein KM, Skurnick J, Wilson DJ, de la Torre AN, Merchant A, Arora R, Samanta AK. Ischemic preconditioning in deceased donor liver transplantation: a prospective randomized clinical trial of safety and efficacy. *Liver Transpl* 2005; **11**: 196-202

106 **Azoulay D**, Lucidi V, Andreani P, Maggi U, Sebagh M, Ichai P, Lemoine A, Adam R, Castaing D. Ischemic preconditioning for major liver resection under vascular exclusion of the liver preserving the caval flow: a randomized prospective study. *J Am Coll Surg* 2006; **202**: 203-211

107 **Jassem W**, Fuggle SV, Cerundolo L, Heaton ND, Rela M. Ischemic preconditioning of cadaver donor livers protects allografts following transplantation. *Transplantation* 2006; **81**: 169-174

108 **Cescon M**, Grazi GL, Grassi A, Ravaioli M, Vetrone G, Ercolani G, Varotti G, D'Errico A, Ballardini G, Pinna AD. Effect of ischemic preconditioning in whole liver transplantation from deceased donors. A pilot study. *Liver Transpl* 2006; **12**: 628-635

109 **Ignarro LJ**, Napoli C, Loscalzo J. Nitric oxide donors and cardiovascular agents modulating the bioactivity of nitric oxide: an overview. *Circ Res* 2002; **90**: 21-28

110 **Kuroki I**, Miyazaki T, Mizukami I, Matsumoto N, Matsumoto I. Effect of sodium nitroprusside on ischemia-reperfusion injuries of the rat liver. *Hepatogastroenterology* 2004; **51**: 1404-1407

111 **Morisue A**, Wakabayashi G, Shimazu M, Tanabe M, Mukai M, Matsumoto K, Kawachi S, Yoshida M, Yamamoto S, Kitajima M. The role of nitric oxide after a short period of liver ischemia-reperfusion. *J Surg Res* 2003; **109**: 101-109

112 **Ghalayini IF**. Nitric oxide-cyclic GMP pathway with some emphasis on cavernosal contractility. *Int J Impot Res* 2004; **16**: 459-469

113 **García-Criado FJ**, Rodriguez-Barca P, García-Cenador MB, Rivas-Elena JV, Grande MT, Lopez-Marcos JF, Mourelle M, López-Novoa JM. Protective effect of new nitrosothiols on the early inflammatory response to kidney ischemia/reperfusion and transplantation in rats. *J Interferon Cytokine Res* 2009; **29**: 441-450

114 **Duranski MR**, Greer JJ, Dejam A, Jaganmohan S, Hogg N, Langston W, Patel RP, Yet SF, Wang X, Kevil CG, Gladwin MT, Lefer DJ. Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. *J Clin Invest* 2005; **115**: 1232-1240

115 **Lu P**, Liu F, Yao Z, Wang CY, Chen DD, Tian Y, Zhang JH, Wu YH. Nitrite-derived nitric oxide by xanthine oxidoreductase protects the liver against ischemia-reperfusion injury. *Hepatobiliary Pancreat Dis Int* 2005; **4**: 350-355

116 **Tripatara P**, Patel NS, Webb A, Rathod K, Lecomte FM, Mazzon E, Cuzzocrea S, Yaqoob MM, Ahluwalia A, Thiemermann C. Nitrite-derived nitric oxide protects the rat kidney against ischemia/reperfusion injury in vivo: role for xanthine oxidoreductase. *J Am Soc Nephrol* 2007; **18**: 570-580

117 **Lauer T**, Preik M, Rassaf T, Strauer BE, Deussen A, Feelisch M, Kelm M. Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. *Proc Natl Acad Sci USA* 2001; **98**: 12814-12819

118 **Nagababu E**, Ramasamy S, Abernethy DR, Rifkind JM. Active nitric oxide produced in the red cell under hypoxic conditions by deoxyhemoglobin-mediated nitrite reduction. *J Biol Chem* 2003; **278**: 46349-46356

119 **Zweier JL**, Wang P, Samouilov A, Kuppusamy P. Enzyme-independent formation of nitric oxide in biological tissues. *Nat Med* 1995; **1**: 804-809

120 **Zhang Z**, Naughton DP, Blake DR, Benjamin N, Stevens CR, Winyard PG, Symons MC, Harrison R. Human xanthine oxidase converts nitrite ions into nitric oxide (NO). *Biochem Soc Trans* 1997; **25**: 524S

121 **Godber BL**, Doel JJ, Sapkota GP, Blake DR, Stevens CR, Eisenthal R, Harrison R. Reduction of nitrite to nitric oxide catalyzed by xanthine oxidoreductase. *J Biol Chem* 2000; **275**: 7757-7763

S- Editor Sun H **L- Editor** Rutherford A **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2890

World J Gastroenterol 2011 June 28; 17(24): 2890-2896
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2011 Baishideng. All rights reserved.

TOPIC HIGHLIGHT

Marcela Kopacova, Associate Professor, MD, PhD, *Series Editor*

Probiotics in hepatology

Jan Lata, Jana Jurankova, Marcela Kopacova, Petr Vitek

Jan Lata, Petr Vitek, Department of Internal Medicine, Faculty of Medicine and University Hospital, Ostrava University, 708 52 Ostrava-Poruba, Czech Republic
Jana Jurankova, Department of Clinical Microbiology, University Hospital Brno, 637 00 Brno, Czech Republic
Marcela Kopacova, 2nd Department of Medicine, Charles University in Praha, Faculty of Medicine at Hradec Kralove, University Teaching Hospital, 500 05 Hradec Králove, Czech Republic
Author contributions: All authors contributed equally to this work.
Supported by Research Grant MSM 6198959223, Ministry of Education, Czech Republic
Correspondence to: Jan Lata, MD, PhD, Professor, Department of Internal Medicine, Faculty of Medicine and University Hospital, Ostrava University, Syllabova 19, 703 00 Ostrava, Czech Republic. jan.lata@osu.cz
Telephone: +420-597091013 Fax: +420-596113146
Received: December 13, 2010 Revised: February 18, 2011
Accepted: February 25, 2011
Published online: June 28, 2011

Abstract

The paper provides a basic review of intestinal microflora and its importance in liver diseases. The intestinal microflora has many important functions, above all to maintain the microbial barrier against established as well as potential pathogens. Furthermore, it influences the motility and perfusion of the intestinal wall, stimulates the intestinal immune system and therefore also the so-called common mucosal immune system, reducing bacterial translocation and producing vitamins. Immune homeostasis at mucosal level results from a controlled response to intestinal luminal antigens. In liver cirrhosis, there are many changes in its function, mostly an increase in bacterial overgrowth and translocation. In this review, probiotics and their indications in hepatology are generally discussed. According to recent knowledge, these preparations are indicated in clinical practice only for cases of hepatic encephalopathy. Probiotics are able to decrease the permeability of the intestinal wall, and decrease bacterial translocation and endotoxemia in animal models as well as in clinical studies, which is extremely important in the prevention of complications of liver cirrhosis and infection after liver transplantation. Probiotics could limit oxidative and inflammatory liver damage and, in some situations, improve the histological state, and thus non-alcoholic steatohepatitis could be considered as another possible indication.

© 2011 Baishideng. All rights reserved.

Key words: Intestinal microflora; Probiotics; Liver encephalopathy; Non-alcoholic steatohepatitis; Liver cirrhosis

Peer reviewers: Akihito Tsubota, Assistant Professor, Institute of Clinical Medicine and Research, Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan; Antonio Gasbarrini, MD, Professor, Department of Internal Medicine, Gemelli Hospital, Catholic University of Rome, Largo A. Gemelli 8, 00168 Rome, Italy

Lata J, Jurankova J, Kopacova M, Vitek P. Probiotics in hepatology. *World J Gastroenterol* 2011; 17(24): 2890-2896 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2890.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2890

INTRODUCTION

In recent years, probiotics have become a promising alternative for the treatment of gastrointestinal and various other diseases. Despite their initially described negative influence on the course of disease, as in the case of acute pancreatitis[1], these medicines are considered safe and their beneficial effects have also been intensively studied and described in hepatology[2].

INTESTINAL MICROFLORA

The average length of an average human adult intestine

is approximately 10 m and its irregular surface is covered with one layer of epithelial cells that represent a surface area of approximately 200 m^2. The intestinal microflora above all plays a very important role in the immune reactions of the body. During fetal development, the intestine is sterile and becomes colonized with the first microorganisms after the passage of the fetus through the birth canal. After birth, the intestine is very quickly colonized with various microorganisms, the composition of which is highly variable during the first few days of life. After the first week, the intestinal microflora achieves a stable composition that depends on the method of birth, environment and type of nutrition. In breast-fed infants, there is a predominance of bifidobacteria, while in infants on milk formula, the number of bifidobacteria can be several times lower. Breast-fed infants are therefore colonized sooner with bacterial strains whose composition resembles that of the intestinal microflora of an adult[3]. The initial colonization of the intestine also plays a very important role in further development of the individual, as the bacteria present may modulate gene expression of epithelial cells and thus create a favorable environment for themselves[4]. The primary colonizers are permanently settled in the intestine and determine intestinal colonization with further bacterial strains later in life, which are important for the final composition of intestinal microflora in adulthood.

Major changes in the features of the intestinal ecosystem occur after weaning[5]. During this period, anaerobic bacteria such as *Bacteroides spp*. and *Clostridium spp*. achieve a strong position and the intestinal ecosystem evolves into its stable form. The intestinal microflora contains a large amount of microbes that weigh more than 1 kg; this quantity exceeds the number of cells in the human body 10-fold. The microbial community of the intestine consists of more than 500 species, most of which have not been cultivated, and many that have not been identified so far. The intestinal microflora contains both bacteria that are fixed in the intestine (autochthonous, resident) and bacteria that only pass through the intestine (transient allochthonous)[6]. Most of the bacteria in the intestine form an anaerobic bioreactor that helps to digest difficult polysaccharides and synthesizes micronutrients including vitamins and short-chain fatty acids. The fermentation products of these bacteria can provide up to 10% of the daily energy needed by an individual[7]. The composition of human gastrointestinal microflora is given in Table 1.

The relationships between the host and their microflora bacteria also play an important role in postnatal development, maturation of the intestine and development of the mucosal immune system.

The intestinal microflora has many important functions, in particular to maintain the microbial barrier against established as well as potential pathogens, and furthermore, it influences the motility and perfusion of the intestinal wall, stimulates the intestinal immune system, and therefore also the so-called common mucosal immune system, reducing bacterial translocation and producing vitamins.

The digestive tract microflora is continuously influenced by numerous physical, chemical and biological factors that can affect its balance, and therefore it represents a constant potential source of digestive tract and whole-body disease. Changes in the total amount, localization, strain or species structure and in the metabolic activity of microorganisms may occur. Impairment of digestive tract microflora physiology can lead to disease or act as its cofactor (infectious, medication-associated, post-antibiotic and post-radiation diarrhea and colitis, functional diseases of the digestive tract-chronic constipation, irritable bowel syndrome, inflammatory bowel diseases, immunodeficiencies, colorectal carcinoma, some extraintestinal diseases and last, but not least, also liver diseases)[8]. In patients with liver cirrhosis, abnormal colonization of the small intestine with colonic bacteria has been reported, while the amount of these bacteria in the small intestine of healthy individuals is small. There is a reciprocal regulatory activity between intestinal microflora and the motility of the small intestine, where the motility is regulated by the presence of intestinal bacteria. Inhibition of gastric acid production induces bacterial overgrowth in the small intestine, whereas the overgrowth of its proximal part correlates with bacterial translocation into the extraintestinal space, such as the mesenteric lymph nodes, liver and spleen.

CHANGES IN INTESTINAL MICROFLORA IN PATIENTS WITH CHRONIC LIVER DISEASES

Intestinal bacterial overgrowth was described in approximately one-third of patients with alcoholic cirrhosis, ascites or advanced liver dysfunction. The main causes are considered to be anchylosis and hypochlorhydrosis, a decrease in IgA secretion and malnutrition caused by liver dysfunction, and possibly alcoholism. Also, the decrease in intestinal motility associated with cirrhotic liver damage facilitates bacterial overgrowth in the small intestine. The impaired immune mechanisms of the mucous membrane of the small intestine facilitating bacterial overgrowth can be one explanation of the repeated and common infections in patients with liver cirrhosis. In particular, the spontaneous infection of ascites-spontaneous bacterial peritonitis (SBP)-is a frequent and severe condition[9,10]. In contrast, suppression or eradication of intestinal facultative anaerobic gram-negative bacteria prevents their translocation and SBP, both in cirrhotic rats and in liver cirrhosis patients.

As a result of bacterial overgrowth, bacterial translocation may occur, and portal hypertension also plays an important role. It leads to vasodilation of the intestinal mucous membrane, edema of the lamina propria, fibromuscular proliferation and hypertrophy of the muscularis mucosae. Furthermore, the integrity of the intestinal mucous membrane is compromised; toxic influences of alcohol, disturbances in biliary secretion, malnutrition, decrease in growth factor secretion (insulin-like growth factor Ⅰ),

Table 1 Composition of the human gastrointestinal tract microflora (from Nord and Kager, 1984)

Microorganisms	Numbers of microorganisms (CFU/mL or CFU/g)			
	Stomach	Jejunum	Ileum	Colon
Total bacterial count	0-10^3	0-10^5	10^3-10^9	10^{10}-10^{12}
Aerobically growing agents				
Family *enterobacteriaceae*	0-10^2	0-10^3	10^2-10^7	10^4-10^{10}
Streptococci	0-10^3	0-10^4	10^2-10^6	10^5-10^{10}
Staphylococci	0-10^2	0-10^3	10^2-10^5	10^4-10^9
Lactobacilli	0-10^3	0-10^4	10^2-10^3	10^6-10^{10}
Yeasts	0-10^3	0-10^2	10^2-10^4	10^4-10^6
Anaerobic bacteria				
Bacteroides	Rare	0-10^3	10^3-10^7	10^{10}-10^{12}
Bifidobacteria	Rare	0-10^4	10^3-10^9	10^4-10^{11}
Peptostreptococci	Rare	0-10^3	10^2-10^6	10^{10}-10^{12}
Clostridia	Rare	Rare	10^2-10^4	10^6-10^{11}
Eubacteria	Rare	Rare	Rare	10^{10}-10^{12}

changes in bile composition and flow or increased levels of nitric oxide may be present, as well as portal hypertension. The increase in intestinal permeability is conspicuously proportionate to the degree of portal hypertension, but it is independent of severity and etiology of liver impairment[11]. In patients with liver cirrhosis and portal hypertension, vascular resistance decreases and splanchnic flow increases. These changes lead to hypoperfusion and hypoxia of the mucous membrane further compromising the vascular wall. As a consequence of this, translocation of intestinal bacteria occurs easily[12].

The term bacterial translocation was used for the first time in 1979[13]. Bacterial translocation is defined as active or passive penetration of live microorganisms and their toxic products across the epithelial layer of the mucous membrane to the lamina propria mucosae. Microorganisms then migrate to the lymph nodes and/or into extraintestinal locations. Under normal conditions, this refers to the small amount of bacteria that are destroyed by the immune system of the lamina propria. Translocation is only possible when there are a high number of bacteria; the literature reports up to 10^8 bacteria in 1 g of stool[14].

Bacteria that escape phagocytes, as well as destruction by complement, can reach the circulation. *Enterobacteria, staphylococci* and *enterococci* are able to translocate, i.e. pass alive across the intestinal epithelium into the mesenteric lymph nodes, blood and other organs, while most other anaerobic microorganisms lack this ability. Bacterial translocation can be verified by positive cultivation from mesenteric lymph nodes. The main mechanisms leading to translocation include a deficit in the local immune response of the mucous membrane, a decrease in phagocytic activity of macrophages as well as neutrophils, an increase in the permeability of the intestinal barrier, and intestinal bacterial overgrowth[15].

Factors that influence bacterial translocation can be divided into 3 groups. These are the bacterial factor, comprising the nature of the translocating agent and the status of the surrounding physiological microflora, the morphological and functional state of the intestinal wall, and not least the so-called defensive factors, i.e. local and systemic antibacterial activities of the organism[16,17]. All of these systems are impaired in patients with liver cirrhosis[18].

PROBIOTICS

The history of probiotics started at the beginning of the last century with Metchnikoff[19]; however, German authors often report a study by Döderlein as the first description of a possible probiotic 16 years before Metchnikoff proposed the use of vaginal lactate-producing bacteria for the inhibition of pathogenic bacteria growth, and attributed the higher average age of certain ethnic groups to the increased intake of fermented milk products and recommended their use.

Probiotics were originally defined as "microorganisms causing growth of other microorganisms", and later on as "live microorganisms that cause or support the beneficial balance of autochthonous microbial population of the gastrointestinal tract (GIT)". These microorganisms do not have to be an essential permanent component of the GIT, but should have a "beneficial influence on the general and health status of an individual". Currently, probiotics are defined more precisely as "monocultures or mixed cultures of live microorganisms that, if administered to a person, positively influence the host by improving the properties of his/her own microflora"[20].

UTILIZATION OF PROBIOTICS IN HEPATOLOGY

In the Cochrane Library Review, there is currently no unambiguous recommendation for administration of probiotics in any indication in hepatology. According to the World Gastroenterology Organisation Practice Guideline "Probiotics and prebiotics" are probiotics in hepatology indicated only for hepatic encephalopathy[21], and in clinical practice, probiotics are now administered in principle only in the above-mentioned treatment of hepatic encephalopathy, with the disadvantage of a higher price compared to the standard treatment. The use of

probiotics in the treatment of non-alcoholic steatohepatitis and in prophylaxis of infections, or some complications in patients with liver cirrhosis, can be expected in the future.

Liver encephalopathy

It is believed that gut-produced ammonia plays a key role in the pathogenesis of hepatic encephalopathy because of the failure of the diseased liver to clear toxic products. Small intestinal overgrowth and delayed gastrointestinal transit time in cirrhotic patients plays an important role[22].

Lactulose and non-absorbable antibiotics currently hold a dominant position in the treatment of liver encephalopathy. One of the effects of lactulose may be a probiotic effect on lactobacilli that reduce the activity of bacterial ureases, resulting in a decrease in hyperammonemia. Probiotics can also have a similar effect and are already included in some recommendations for the treatment of minimal liver encephalopathy[23].

As early as the 1960s, the beneficial effect of *Lactobacillus acidophilus* was described on the course of liver encephalopathy in patients with liver cirrhosis[24]. In a more recent study on 97 patients, the beneficial effect of a synbiotic (mixture of a probiotic and prebiotic) on minimal liver encephalopathy was observed, with a decrease in ammonium levels as well as the improvement of symptoms of encephalopathy[25]. Minimal liver encephalopathy is described as an otherwise inexplicable impairment of cognitive functions such as prolonged psychomotor tempo, lack of attention, impairment of fine motor functions and the perception of visual sensations that can only be detected using special neurophysiological tests, and is present in 30%-70% of patients with liver cirrhosis without liver encephalopathy. In the treatment of advanced liver encephalopathy, a beneficial effect of *Enterococcus faecium* was observed, the administration of which led to an improvement in clinical status, electroencephalogram findings and a reduction of ammonium levels[26]; the treatment of the *Enterococcus* strain SF69[27] also had a similar effect. A mixture of probiotics[28] may have an even better effect. A combination of *Bifidobacterium* + fructo-oligosaccharides also demonstrated a significant reduction in the Trail Making Test B, a significant increase in the Symbol Digit Modalities Test and Block Design Test and improvement in some laboratory findings[29].

A study confirming improvement of liver encephalopathy during long-term administration of probiotic yogurts with the advantage of excellent adherence with potential for long-term adherence[30] is also of interest.

So far, there are only very few experimental studies, however, the studies comparing the efficacy of probiotic preparation *Golden Bifid* and lactulose on an experimental rat model of minimal hepatic encephalopathy induced by thioactamide showed excellent effects in lowering the level of ammonemia and endotoxemia, improving hepatic histopathology of rats, and decreasing the incidence of minimal hepatic encephalopathy[31].

The use of probiotics in common clinical practice as well as evaluation of the economic effect of the treatment will, however, require further studies. At the moment, a study titled "Probiotic *Lactobacillus* GG (LGG) in Patients with Minimal Hepatic Encephalopathy" is being conducted at Virginia Commonwealth University and we can only hope it will bring novel, positive results.

Effects on bacterial intestinal translocation, reduction in infections or prophylaxis of liver cirrhosis complications

Infectious complications are very frequently caused by bacterial strains that originate from the digestive tract. Bacterial overgrowth, as well as the associated translocation of microbes across the dysfunctional mucous membrane barrier, occurs for the above-mentioned reasons, especially in liver cirrhosis. The small gut of cirrhotic rats is contaminated by colonic microflora, which translocate to mesenteric lymph nodes, and is the most important cause of infection of ascites and spontaneous bacterial peritonitis. The identity of microbial strains in the gut, mesenteric lymph nodes and ascites was demonstrated by analysis of macrorestricted fragments DNA[32].

In 50%-70% of patients with liver cirrhosis, bacterial overgrowth occurs in the small intestine as a result of gram-negative colon microflora contamination. As a result, an impairment of the intestinal barrier with increased bacterial translocation occurs. Apparently, it is the most important point of entry of infection in cirrhotic patients; generally, infections are very common in liver cirrhosis and can be involved in many complications associated with the disease. In the first animal studies with short term application of *Lactobacillus* GG, the treatment did not prevent translocation of colonic microflora, although it was able to colonize the cecum in 90% of cirrhotic rats[33]. In later studies, preventive treatment with *Lactobacillus plantarum* inhibited an increase in permeability after subsequent application of *E. coli*[34], and *Lactobacillus johnsonii* La1 with antioxidants were able to decrease endotoxemia and prevent bacterial translocation in cirrhotic rats[35].

In clinical studies, administration of a symbiotic reduced the endotoxemia, which is an indicator of the degree of translocation[11]. In our study, a reduction in endotoxemia was achieved through administration of *E. coli* Nissle for 42 d[36]. The clinical significance of this has yet to be verified, but the beneficial influence on the prophylaxis of severe infectious complications, such as spontaneous bacterial peritonitis, can be expected.

In both the above-mentioned papers, an improvement in functional liver capabilities was observed, evaluated according to the Child-Pugh classification. It can be presumed that the recovery of physiological microflora in the digestive tract will reduce the liver load of toxic metabolites, above all endotoxin, which might potentially be absorbed. It stimulates secretion of cytokines. The cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 and interleukin-6 can influence the formation and degradation of the extracellular matrix, which is important for the development of liver fibrosis as well as during cirrhotic rearrangement[37]. Apart from the above-

mentioned decrease in endotoxin levels, a direct decrease in cytokine levels after administration of VSL#3 probiotic has also been reported[38]. There are some data showing, that Se-enriched *Lactobacillus* can intervene in carbon tetrachloride-induced liver injury in mice by enhancing macrophage function to maintain normal and beneficial effects, elevating antioxidant-enzyme activities, reducing lipid peroxidation reaction and inhibiting excessive release of TNF-α[39]. However, this extremely important finding will have to be confirmed. Another study with VSL#3, presenting a trend of a reduction in plasma endotoxin, on the other hand showed no change in the hepatic venous pressure gradient or intestinal permeability[40].

In liver cirrhosis of alcoholic etiology, the alcohol itself may play a role, such as increased gut permeability, endotoxemia, and TNF-α production[41]. In rats, *Lactobacillus* GG has been shown to reduce alcohol induced gut leakiness and steatohepatitis[42]. The same group also found that the mucosa-associated microflora was altered in rats on a high alcohol diet, and this dysbiosis could be counteracted by *Lactobacillus* GG or oat supplementation[43]. In a rat model of acute pancreatitis, synbiotic (*Lactobacillus acidophilus*, *Lactobacillus helveticus* and *Bifidobacterium* in an enriched medium) and metronidazole were able to effectively protect against endotoxin/bacterial translocation, as well as liver damage in the course of acute pancreatitis and concomitant heavy alcohol consumption. The beneficial effect of synbiotics on liver histology seems to be correlated with endotoxemia. Metronidazole did not produce such a beneficial effect; in fact, it further worsened liver damage when alcohol was added to the background of ongoing acute pancreatic inflammation[44].

However, no large randomization study has been carried out as yet which would be relevant for clinical practice, although a recent study again confirmed the theoretic presumptions for beneficial action in this field[45]. The "Probiotics for the Prevention of Major Complications of Cirrhosis" study, carried out in the Meir Medical Center in Israel, was finished last year but its results have not yet been published.

Non-alcoholic steatohepatitis

Fatty liver disease that develops in the absence of alcohol abuse is increasingly recognized as a major health burden. Non-alcoholic steatohepatitis (NASH) was first described by Ludwig in 1980 as a disease that histologically mimicked alcoholic hepatitis and that also may progress to cirrhosis[46]. The diagnostic criteria for NASH continue to evolve and rely on the histologic findings of steatosis, hepatocellular injury (ballooning, Mallory bodies), and the pattern of fibrosis in patients with minimal intake of ethanol (< 20 g ethanol/d)[47]. Recently, NASH has been studied extensively as it is relatively frequent, and may lead to the development of liver cirrhosis. So far, treatment has not been established and probiotics may play an important role, as the bacterial overgrowth and associated increase of proinflammatory cytokines are important etiopathogenic mechanisms of NASH[48]. Clinical studies so far have been missing in this field, but the positive effect of probiotics has already been described in some laboratory studies. In mice with non-alcoholic liver steatosis, treatment with probiotics or anti-TNF antibodies improved the histological picture of the degree of damage to the liver parenchyma, led to a decrease in the alkaline phosphatase level, improved insulin resistance, and the content of total fatty acids in the liver also dropped. In another study on a model of non-alcoholic liver steatosis, treatment with the probiotic VSL#3 or TNF-α antibodies had a positive influence on histological findings, the fatty acids in hepatocytes were reduced, the ALT level decreased and the expression of TNF-α[49,50] was reduced. Similar data suggesting that VSL#3 administration could limit oxidative and inflammatory liver damage were published in a more recent study[51]. Some previous studies showed that a high fat diet caused obesity, hepatic steatosis and natural killer T-cell (NKT cell) depletion[52]. The VSL#3 was also shown to increase hepatic NKT cell number and reduce inflammatory signaling[53]. Another study indicated that VSL#3 modulates liver fibrosis but does not protect from inflammation and steatosis in NASH. It means, that VSL#3 effects on fibrosis may occur even in the absence of significant changes in markers of inflammation and fat in the liver[54].

In the Cochrane Library Review, there is currently no unambiguous recommendation for administration of probiotics in NASH, even if the results from pilot studies seem promising. Randomized clinical trials are necessary to assess the clinical implication of probiotic therapy in non-alcoholic fatty liver disease and non-alcoholic steatohepatitis[55]. Two clinical studies on the effects of probiotics on NASH are currently being conducted in Hong Kong and in Israel. Hopefully their conclusions will be encouraging.

Prophylaxis of infections after liver transplantation

In the past, several papers were published that confirmed the positive influence of probiotic administration on postoperative course after large abdominal surgery. The studies are mostly of small sample size and exhibit design flaws, but they showed statistically significant differences in infectious complications in favor of synbiotics[56-58], and the synbiotic group did require significantly less days of antibiotic therapy[59].

Patients in the postoperative period after a liver transplant are mainly at risk of infection by organisms, coming in most cases from the digestive tract. As already discussed in the theoretical part of our paper, the translocation of bacteria or their parts across the intestinal wall into the circulation occurs as a result of disturbances in barrier function of the intestine and disturbance of the immune system. Bacteria that translocated from the intestinal tract can be carried through the circulation into more distant systems and cause colonization or infection in extraintestinal locations. A prospective, randomized, double-blind study was published on 66 patients after liver transplantation, whereas half of the patients received a combination of 4 *Lactobacillus spp*. together with the standard enteral

nutrition. In the probiotic group, a significant reduction in postoperative bacterial infection (3% against 48%) was observed and the length of the antibiotic therapy was substantially reduced[60]. In our study on patients after liver transplantation, we demonstrated a correlation between an increase in endotoxin and the subsequent presence of phenotypically as well as genotypically identical strains (originally cultivated from the gastrointestinal tract) in an extraintestinal location[61]. Monitoring serum endotoxin levels can probably confirm bacterial translocation with an increased risk of infectious complications and this result further confirms the positive effect of probiotics in patients after liver transplantation. However, the conclusion is similar from the perspective of evidence-based medicine-use of prebiotics and probiotics in prevention of bacterial sepsis after liver transplantation is promising. Further randomized clinical trials are necessary.

CONCLUSION

Probiotics are becoming part of numerous therapeutic modalities in hepatology, as their effect on intestinal microflora can positively influence many liver diseases. However, verification of the efficiency of this treatment from the perspective of evidence-based medicine will be difficult, as different probiotics can be expected to have different effects in different diseases. With respect to the increasing number of studies on probiotics, the future prospects in this field are optimistic.

REFERENCES

1 **Besselink MG**, van Santvoort HC, Buskens E, Boermeester MA, van Goor H, Timmerman HM, Nieuwenhuijs VB, Bollen TL, van Ramshorst B, Witteman BJ, Rosman C, Ploeg RJ, Brink MA, Schaapherder AF, Dejong CH, Wahab PJ, van Laarhoven CJ, van der Harst E, van Eijck CH, Cuesta MA, Akkermans LM, Gooszen HG. Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial. *Lancet* 2008; **371**: 651-659

2 **Gratz SW**, Mykkanen H, El-Nezami HS. Probiotics and gut health: a special focus on liver diseases. *World J Gastroenterol* 2010; **16**: 403-410

3 **Yoshioka H**, Iseki K, Fujita K. Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. *Pediatrics* 1983; **72**: 317-321

4 **Hooper LV**, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 2001; **291**: 881-884

5 **Mackie RI**, Sghir A, Gaskins HR. Developmental microbial ecology of the neonatal gastrointestinal tract. *Am J Clin Nutr* 1999; **69**: 1035S-1045S

6 **Saavedra JM**, Tschernia A. Human studies with probiotics and prebiotics: clinical implications. *Br J Nutr* 2002; **87** Suppl 2: S241-S246

7 **Macpherson AJ**, Harris NL. Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol* 2004; **4**: 478-485

8 **Hanson LA**, Yolken RH. In: Probiotics, other nutritional factors and intestinal microflora. Philadelphia: Lippincot-Raven, 1999: 18-67

9 **Koulaouzidis A**, Bhat S, Saeed AA. Spontaneous bacterial peritonitis. *World J Gastroenterol* 2009; **15**: 1042-1049

10 **Lata J**, Stiburek O, Kopacova M. Spontaneous bacterial peritonitis: a severe complication of liver cirrhosis. *World J Gastroenterol* 2009; **15**: 5505-5510

11 **Ersöz G**, Aydin A, Erdem S, Yüksel D, Akarca U, Kumanlioglu K. Intestinal permeability in liver cirrhosis. *Eur J Gastroenterol Hepatol* 1999; **11**: 409-412

12 **Goulis J**, Patch D, Burroughs AK. Bacterial infection in the pathogenesis of variceal bleeding. *Lancet* 1999; **353**: 139-142

13 **Berg RD**, Garlington AW. Translocation of certain indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs in a gnotobiotic mouse model. *Infect Immun* 1979; **23**: 403-411

14 **Guarner C**, Runyon BA, Young S, Heck M, Sheikh MY. Intestinal bacterial overgrowth and bacterial translocation in cirrhotic rats with ascites. *J Hepatol* 1997; **26**: 1372-1378

15 **Ramachandran A**, Balasubramanian KA. Intestinal dysfunction in liver cirrhosis: Its role in spontaneous bacterial peritonitis. *J Gastroenterol Hepatol* 2001; **16**: 607-612

16 **Luyer MD**, Buurman WA, Hadfoune M, Speelmans G, Knol J, Jacobs JA, Dejong CH, Vriesema AJ, Greve JW. Strain-specific effects of probiotics on gut barrier integrity following hemorrhagic shock. *Infect Immun* 2005; **73**: 3686-3692

17 **Rachmilewitz D**, Katakura K, Karmeli F, Hayashi T, Reinus C, Rudensky B, Akira S, Takeda K, Lee J, Takabayashi K, Raz E. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology* 2004; **126**: 520-528

18 **Guarner C**, Soriano G. Bacterial translocation and its consequences in patients with cirrhosis. *Eur J Gastroenterol Hepatol* 2005; **17**: 27-31

19 **Metchnikoff E**. Prolongation of life- Optimistic Studies. New York: Putnam and Sons, 1908

20 **Havenaar R**, Ten Brink B, Huis in't Veld JHJ. Selection of strains for probiotic use. Probiotics: the Scientific Basis) London: Chapman and Hall, 1992: 209-224

21 **Gupta A**, Dhiman RK, Kumari S, Rana S, Agarwal R, Duseja A, Chawla Y. Role of small intestinal bacterial overgrowth and delayed gastrointestinal transit time in cirrhotic patients with minimal hepatic encephalopathy. *J Hepatol* 2010; **53**: 849-855

22 **http://www.worldgastroenterology.org/guidelines/19_probiotics_prebiotics**

23 **Foster KJ**, Lin S, Turck CJ. Current and emerging strategies for treating hepatic encephalopathy. *Crit Care Nurs Clin North Am* 2010; **22**: 341-350

24 **Macbeth WA**, Kass EH, Mcdermott WV Jr. Treatment of hepatic encephalopathy by alteration of intestinal flora with lactobacillus acidophilus. *Lancet* 1965; **1**: 399-403

25 **Liu Q**, Duan ZP, Ha DK, Bengmark S, Kurtovic J, Riordan SM. Synbiotic modulation of gut flora: effect on minimal hepatic encephalopathy in patients with cirrhosis. *Hepatology* 2004; **39**: 1441-1449

26 **Boca M**, Vyskocil M, Mikulecký M, Ebringer L, Kolibás E, Kratochvíl'ová H, Buzgová D. [Complex therapy of chronic hepatic encephalopathy supplemented with probiotic: comparison of two studies]. *Cas Lek Cesk* 2004; **143**: 324-328

27 **Loguercio C**, Del Vecchio Blanco C, Coltorti M. Enterococcus lactic acid bacteria strain SF68 and lactulose in hepatic encephalopathy: a controlled study. *J Int Med Res* 1987; **15**: 335-343

28 **Solga SF**. Probiotics can treat hepatic encephalopathy. *Med Hypotheses* 2003; **61**: 307-313

29 **Malaguarnera M**, Gargante MP, Malaguarnera G, Salmeri M, Mastrojeni S, Rampello L, Pennisi G, Li Volti G, Galvano F. Bifidobacterium combined with fructo-oligosaccharide versus lactulose in the treatment of patients with hepatic encephalopathy. *Eur J Gastroenterol Hepatol* 2010; **22**: 199-206

30 **Bajaj JS**, Saeian K, Christensen KM, Hafeezullah M, Varma RR, Franco J, Pleuss JA, Krakower G, Hoffmann RG, Binion DG. Probiotic yogurt for the treatment of minimal hepatic encephalopathy. *Am J Gastroenterol* 2008; **103**: 1707-1715

31 **Jia L**, Zhang MH. Comparison of probiotics and lactulose

in the treatment of minimal hepatic encephalopathy in rats. *World J Gastroenterol* 2005; **11**: 908-911
32 **Llovet JM**, Bartolí R, March F, Planas R, Viñado B, Cabré E, Arnal J, Coll P, Ausina V, Gassull MA. Translocated intestinal bacteria cause spontaneous bacterial peritonitis in cirrhotic rats: molecular epidemiologic evidence. *J Hepatol* 1998; **28**: 307-313
33 **Bauer TM**, Fernández J, Navasa M, Vila J, Rodés J. Failure of Lactobacillus spp. to prevent bacterial translocation in a rat model of experimental cirrhosis. *J Hepatol* 2002; **36**: 501-506
34 **Mangell P**, Nejdfors P, Wang M, Ahrné S, Weström B, Thorlacius H, Jeppsson B. Lactobacillus plantarum 299v inhibits Escherichia coli-induced intestinal permeability. *Dig Dis Sci* 2002; **47**: 511-516
35 **Chiva M**, Soriano G, Rochat I, Peralta C, Rochat F, Llovet T, Mirelis B, Schiffrin EJ, Guarner C, Balanzó J. Effect of Lactobacillus johnsonii La1 and antioxidants on intestinal flora and bacterial translocation in rats with experimental cirrhosis. *J Hepatol* 2002; **37**: 456-462
36 **Lata J**, Novotný I, Príbramská V, Juránková J, Fric P, Kroupa R, Stibůrek O. The effect of probiotics on gut flora, level of endotoxin and Child-Pugh score in cirrhotic patients: results of a double-blind randomized study. *Eur J Gastroenterol Hepatol* 2007; **19**: 1111-1113
37 **Wu CD**, Li MZ, Chen CL. Endotoxin-induced liver injury and plasma tumor necrosis factor alfa, interleukin 6 level changes in rabbits. *Chin J Dig* 1995; **15**: 256-258
38 **Loguercio C**, Federico A, Tuccillo C, Terracciano F, D'Auria MV, De Simone C, Del Vecchio Blanco C. Beneficial effects of a probiotic VSL#3 on parameters of liver dysfunction in chronic liver diseases. *J Clin Gastroenterol* 2005; **39**: 540-543
39 **Chen L**, Pan DD, Zhou J, Jiang YZ. Protective effect of selenium-enriched Lactobacillus on CCl4-induced liver injury in mice and its possible mechanisms. *World J Gastroenterol* 2005; **11**: 5795-5800
40 **Tandon P**, Moncrief K, Madsen K, Arrieta MC, Owen RJ, Bain VG, Wong WW, Ma MM. Effects of probiotic therapy on portal pressure in patients with cirrhosis: a pilot study. *Liver Int* 2009; **29**: 1110-1115
41 **Kirpich IA**, Solovieva NV, Leikhter SN, Shidakova NA, Lebedeva OV, Sidorov PI, Bazhukova TA, Soloviev AG, Barve SS, McClain CJ, Cave M. Probiotics restore bowel flora and improve liver enzymes in human alcohol-induced liver injury: a pilot study. *Alcohol* 2008; **42**: 675-682
42 **Forsyth CB**, Farhadi A, Jakate SM, Tang Y, Shaikh M, Keshavarzian A. Lactobacillus GG treatment ameliorates alcohol-induced intestinal oxidative stress, gut leakiness, and liver injury in a rat model of alcoholic steatohepatitis. *Alcohol* 2009; **43**: 163-172
43 **Mutlu E**, Keshavarzian A, Engen P, Forsyth CB, Sikaroodi M, Gillevet P. Intestinal dysbiosis: a possible mechanism of alcohol-induced endotoxemia and alcoholic steatohepatitis in rats. *Alcohol Clin Exp Res* 2009; **33**: 1836-1846
44 **Marotta F**, Barreto R, Wu CC, Naito Y, Gelosa F, Lorenzetti A, Yoshioka M, Fesce E. Experimental acute alcohol pancreatitis-related liver damage and endotoxemia: synbiotics but not metronidazole have a protective effect. *Chin J Dig Dis* 2005; **6**: 193-197
45 **Stadlbauer V**, Mookerjee RP, Hodges S, Wright GA, Davies NA, Jalan R. Effect of probiotic treatment on deranged neutrophil function and cytokine responses in patients with compensated alcoholic cirrhosis. *J Hepatol* 2008; **48**: 945-951
46 **Ludwig J**, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980; **55**: 434-438
47 **Neuschwander-Tetri BA**, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. *Hepatology* 2003; **37**: 1202-1219
48 **Medina J**, Fernández-Salazar LI, García-Buey L, Moreno-Otero R. Approach to the pathogenesis and treatment of nonalcoholic steatohepatitis. *Diabetes Care* 2004; **27**: 2057-2066
49 **Fedorak RN**, Madsen KL. Probiotics and prebiotics in gastrointestinal disorders. *Curr Opin Gastroenterol* 2004; **20**: 146-155
50 **Li Z**, Yang S, Lin H, Huang J, Watkins PA, Moser AB, Desimone C, Song XY, Diehl AM. Probiotics and antibodies to TNF inhibit inflammatory activity and improve nonalcoholic fatty liver disease. *Hepatology* 2003; **37**: 343-350
51 **Esposito E**, Iacono A, Bianco G, Autore G, Cuzzocrea S, Vajro P, Canani RB, Calignano A, Raso GM, Meli R. Probiotics reduce the inflammatory response induced by a high-fat diet in the liver of young rats. *J Nutr* 2009; **139**: 905-911
52 **Li Z**, Soloski MJ, Diehl AM. Dietary factors alter hepatic innate immune system in mice with nonalcoholic fatty liver disease. *Hepatology* 2005; **42**: 880-885
53 **Ma X**, Hua J, Li Z. Probiotics improve high fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells. *J Hepatol* 2008; **49**: 821-830
54 **Velayudham A**, Dolganiuc A, Ellis M, Petrasek J, Kodys K, Mandrekar P, Szabo G. VSL#3 probiotic treatment attenuates fibrosis without changes in steatohepatitis in a diet-induced nonalcoholic steatohepatitis model in mice. *Hepatology* 2009; **49**: 989-997
55 **Lirussi F**, Mastropasqua E, Orando S, Orlando R. Probiotics for non-alcoholic fatty liver disease and/or steatohepatitis. *Cochrane Database Syst Rev* 2007; CD005165
56 **Kanazawa H**, Nagino M, Kamiya S, Komatsu S, Mayumi T, Takagi K, Asahara T, Nomoto K, Tanaka R, Nimura Y. Synbiotics reduce postoperative infectious complications: a randomized controlled trial in biliary cancer patients undergoing hepatectomy. *Langenbecks Arch Surg* 2005; **390**: 104-113
57 **Sugawara G**, Nagino M, Nishio H, Ebata T, Takagi K, Asahara T, Nomoto K, Nimura Y. Perioperative synbiotic treatment to prevent postoperative infectious complications in biliary cancer surgery: a randomized controlled trial. *Ann Surg* 2006; **244**: 706-714
58 **Reddy BS**, Macfie J, Gatt M, Larsen CN, Jensen SS, Leser TD. Randomized clinical trial of effect of synbiotics, neomycin and mechanical bowel preparation on intestinal barrier function in patients undergoing colectomy. *Br J Surg* 2007; **94**: 546-554
59 **Rayes N**, Hansen S, Seehofer D, Müller AR, Serke S, Bengmark S, Neuhaus P. Early enteral supply of fiber and Lactobacilli versus conventional nutrition: a controlled trial in patients with major abdominal surgery. *Nutrition* 2002; **18**: 609-615
60 **Rayes N**, Seehofer D, Theruvath T, Schiller RA, Langrehr JM, Jonas S, Bengmark S, Neuhaus P. Supply of pre- and probiotics reduces bacterial infection rates after liver transplantation--a randomized, double-blind trial. *Am J Transplant* 2005; **5**: 125-130
61 **Jurankova J**, Příbramska V, Lata J, Sauer P, Stosova T, Studenik P, Mejzlik V, Skacelova H, Koukalova D. Demonstration of bacterial translocation after liver transplantation - first results. *Ces a Slov Gastroent a Hepatol* 2008; **62**: 253-258

S- Editor Tian L **L- Editor** Cant MR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2897

World J Gastroenterol 2011 June 28; 17(24): 2897-2908
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2011 Baishideng. All rights reserved.

TOPIC HIGHLIGHT

Marcela Kopacova, Associate Professor, MD, PhD, *Series Editor*

Molecular biology of pancreatic cancer

Miroslav Zavoral, Petra Minarikova, Filip Zavada, Cyril Salek, Marek Minarik

Miroslav Zavoral, Petra Minarikova, Filip Zavada, Charles University, 1st Medical Faculty, Internal Clinic, 128 00 Prague 2, Czech Republic
Miroslav Zavoral, Petra Minarikova, Filip Zavada, Central Military Hospital, 162 00 Prague 6, Czech Republic
Miroslav Zavoral, Petra Minarikova, Filip Zavada, Institute for postgraduate medical education, 141 00 Prague 4, Czech Republic
Cyril Salek, Institute of hematology and blood transfusion, 128 00 Prague 2, Czech Republic
Marek Minarik, Center for Applied Genomics of Solid Tumors (CEGES), Genomac International, 155 41 Prague 6, Czech Republic
Marek Minarik, Laboratory for Molecular Genetics and Oncology, Genomac International, 155 41 Prague 5, Czech Republic
Author contributions: Zavoral M performed the data analysis and literature search for the chronic pancreatitis, diabetes mellitus and molecular diagnostics sections; Minarikova P performed the data analysis and literature search for the risk factors and genetic susceptibility sections; Zavada F performed the data analysis and literature search for the molecular diagnostics section; Salek C performed the data analysis and literature search for the epidemiology and chronic pancreatitis sections; Minarik M contributed to the genetic susceptibility section, and performed the data analysis and literature search for the molecular mechanisms section; Zavoral M and Minarik M wrote the paper; Zavoral M, Minarikova P, Zavada F, Salek C and Minarik M revised and approved the final draft of the paper.
Supported by the Czech Ministry of Health Project 9809. It is a contribution No. 3 from CEGES (OPPK CZ.2.16/3.1.00/22213)
Correspondence to: Marek Minarik, PhD, Head, CEGES and Laboratory for Molecular Genetics and Oncology, Genomac International., Bavorska 856, 155 41 Prague 5, Czech Republic. mminarik@email.com
Telephone: +42-224-458048 Fax: +42-224-458021
Received: January 6, 2011 Revised: February 19, 2011
Accepted: February 26, 2011
Published online: June 28, 2011

Abstract

In spite of continuous research efforts directed at early detection and treatment of pancreatic cancer, the outlook for patients affected by the disease remains dismal. With most cases still being diagnosed at advanced stages, no improvement in survival prognosis is achieved with current diagnostic imaging approaches. In the absence of a dominant precancerous condition, several risk factors have been identified including family history, chronic pancreatitis, smoking, diabetes mellitus, as well as certain genetic disorders such as hereditary pancreatitis, cystic fibrosis, familial atypical multiple mole melanoma, and Peutz–Jeghers and Lynch syndromes. Most pancreatic carcinomas, however, remain sporadic. Current progress in experimental molecular techniques has enabled detailed understanding of the molecular processes of pancreatic cancer development. According to the latest information, malignant pancreatic transformation involves multiple oncogenes and tumor-suppressor genes that are involved in a variety of signaling pathways. The most characteristic aberrations (somatic point mutations and allelic losses) affect oncogenes and tumor-suppressor genes within RAS, AKT and Wnt signaling, and have a key role in transcription and proliferation, as well as systems that regulate the cell cycle (SMAD/DPC, CDKN2A/p16) and apoptosis (TP53). Understanding of the underlying molecular mechanisms should promote development of new methodology for early diagnosis and facilitate improvement in current approaches for pancreatic cancer treatment.

© 2011 Baishideng. All rights reserved.

Key words: Pancreatic cancer; Risk factors; Molecular biology; Pancreatitis; Diabetes

Peer reviewers: Jose JG Marin, Professor, Head of the Departament of Physiology and Pharmacology, University of Salamanca, CIBERehd, Campus Miguel de Unamuno, ED-S09, Salamanca 37007, Spain

Zavoral M, Minarikova P, Zavada F, Salek C, Minarik M. Molecular biology of pancreatic cancer. *World J Gastroenterol* 2011; 17(24): 2897-2908 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2897.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2897

INTRODUCTION

Pancreatic cancer is a fatal illness that is characterized by late diagnosis in the absence of early symptoms, which results in identification of the illness at an advanced stage. Despite all therapeutic efforts, the mortality of pancreatic cancer remains high, with the number of newly diagnosed patients nearly equaling that of deceased patients[1].

The incidence of pancreatic cancer is constantly on the rise, especially in regions of North America, Europe and Japan. In the United States, pancreatic cancer represents the fourth most frequent cause of death from cancer. In Europe it ranks fifth. In 2009, 42 470 new cases of this disease were diagnosed, and in the same year, 35 240 patients died of this disease[2]. One of the highest incidence and mortality rates among EU countries is observed in the Czech Republic, which, according to GLOBOSCAN 2008 data, ranks second in incidence (Figure 1A) and first in mortality (Figure 1B), followed by Hungary and Finland. In the Czech Republic, pancreatic cancer is the 10th most frequent malignancy, and accounts for 2.6% of all newly diagnosed neoplasms. In 2005, the absolute number of newly diagnosed pancreatic cancer cases was 901 in men and 876 in women. In that same year, the absolute number of deceased persons from the disease reached 1580 (819 men and 761 women)[3].

Five-year survival for pancreatic cancer is < 5%, mainly due to its late diagnosis, when it is already at an advanced stage[4]. At the time of diagnosis, < 5% of tumors are resectable. Median survival following surgical resection ranges from 13 to 21 mo. Without surgery, median survival is a mere 2.5-8 mo[2].

RISK FACTORS

Due to the relative rarity of pancreatic cancer, which is characterized by a complexity of underlying carcinogenesis, it is likely that a combination of multiple factors contributes to the initiation of the disease. Some factors, such as smoking or alcohol intake, can be controlled, while others such as age or family history cannot. Although most risk factors do not directly cause the disease, level of exposure often influences cancer development. As the treatment options are still limited and the survival prognosis remains poor, identification and evasion of the controllable risk factors becomes particularly important for individuals at high risk[5]. The most prominent pancreatic cancer risk factors are summarized in Table 1.

Smoking and alcohol

Cigarette smoking represents one of the most significant and most widely studied risk factors for pancreatic cancer. The carcinogenic effect of tobacco smoke on pancreatic tissue is explained as the direct action of N-nitrosamines or their secretion into bile and their subsequent reflux into the pancreatic duct. Active smoking

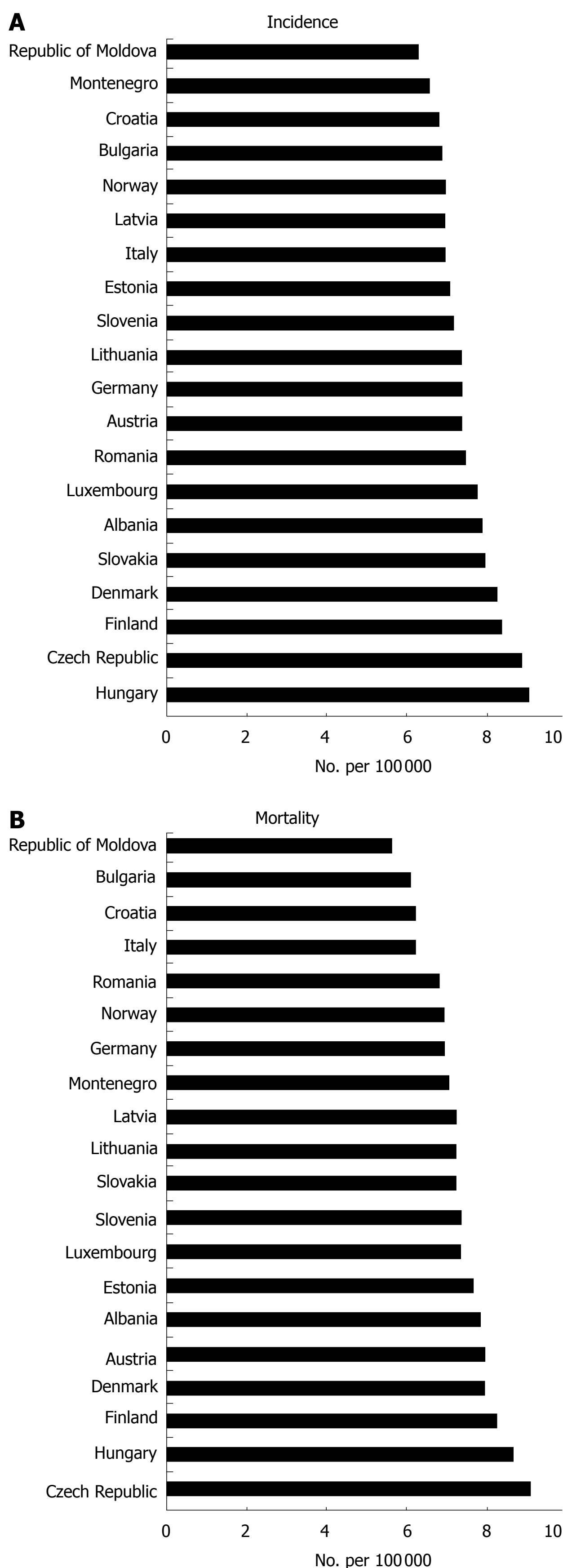


Figure 1 Standardized incidence (A) and mortality (B) rates for the 20 European countries with the highest occurrence of pancreatic cancer (Adapted from GLOBOCAN 2008).

Table 1 Pancreatic cancer risk factors

Factor	Type	Group	Maximum risk to average ratio
Smoking	Exogenous	Behavioral	3
Alcohol	Exogenous	Behavioral	Non-significant
Diet/obesity	Exogenous	Behavioral	1.72
Occupational hazard	Exogenous	Environmental	-
Radiation	Exogenous	Environmental	Inconclusive
Age	Endogenous	Biological	-
Race	Endogenous	Biological/ behavioral/ environmental	1.4
Family history	Endogenous	Genetic	32
Peutz-Jeghers syndrome	Endogenous	Genetic	132
FAMMM syndrome	Endogenous	Genetic	13.1
HNPCC	Endogenous	Genetic	7
Hereditary pancreatitis	Endogenous	Genetic	60
Chronic pancreatitis	Endogenous	Behavioral	26.3
Diabetes mellitus	Endogenous	Biological/ environmental	2.0
Hormonal	Endogenous	Biological	Inconclusive
Allergy	Endogenous	Biological	Non-significant

FAMMM: Familial atypical multiple mole melanoma; HNPCC: Hereditary non-polyposis colorectal carcinoma syndrome.

increases the relative risk of pancreatic cancer 1.5-3-fold, depending on the number of cigarettes smoked and the duration of this habit. Passive smoking has not been shown to be a risk factor[6].

A number of epidemiological studies have focused on the relationship between alcohol consumption and development of pancreatic cancer. An analysis of 14 prospective studies has not confirmed any association between alcohol consumption and a higher risk of pancreatic cancer[3]. Only a very weak association has been demonstrated in the case of alcohol consumption at a dose of ≥ 30 g/d, regardless of the alcohol source of beer, wine or spirits. There is also a significant association with body mass index (BMI), whereby a slight increase in cancer risk has been described in persons with an alcohol consumption of ≥ 30 g/d and a BMI of ≤ 25 kg/m^2. From the aforementioned, it is possible to infer that the association between alcohol consumption and development of pancreatic cancer is only implied.

This association is apparently conditional on the development of chronic pancreatitis, for which alcohol is a known inducer, and chronic pancreatitis is an independent risk factor for this cancer. It thus appears that alcohol consumption at lower doses that do not damage pancreatic tissue does not carry a higher risk for developing pancreatic cancer.

Diet and obesity

Based on a number of relevant studies, it is possible to observe an association with a diet rich in animal fats and higher consumption of meat (roasted, grilled or fried). The results of studies that focus on the effect of cholesterol, eggs, milk and dairy product consumption on increased risk of pancreatic cancer are inconsistent[7]. In contrast, a diet rich in fiber, fruit, vegetables and vitamins, especially vitamin C, is considered to be a protective factor[8]. Omega-3 unsaturated fatty acids that are contained mainly in fish oil also act protectively. A similar protective effect has been shown for substances that influence DNA methylation and synthesis, such as folates. As demonstrated in an analysis of 14 prospective studies, a positive association between alcohol consumption and cancer has been discovered for alcoholics with a low daily folate intake. Similar results have also been observed in the case of methionine. No association with coffee or tea consumption has been demonstrated[3].

Obesity is a generally recognized risk factor for pancreatic cancer in men and women[9]. In cases in which prospective studies have evaluated BMI, men with BMI ≥ 30 kg/m^2 had a higher relative risk compared to women with the same BMI[10]. One particularly interesting fact is that physical activity did not reduce the risk in persons with BMI < 25 kg/m^2, but it was indirectly proportional to the risk in persons with BMI ≥ 25 kg/m^2. Physical activity, which in its final effect leads to increased insulin resistance, decreases the degree of risk in obese patients.

Occupational hazards

The possible influence of aromatic and heterocyclic amines as well as exposure to chlorinated solvents in the carcinogenesis of pancreatic cancer remains unclear. The groups most at risk from this aspect include workers in the petrochemical and rubber industry, as well as barbers and hairdressers in whom exposure to these substances is higher compared to the general population[11]. In contrast, the influence of heavy metals and especially cadmium, in view of its accumulation in pancreatic tissue, has demonstrated an accentuation of neoplastic processes. Another element that is suspected of a carcinogenic effect on pancreatic tissue is chromium. Nonetheless, occupational exposure leads only to an imperceptible increase in the relative risk, in view of the minimal doses involved.

Radiation

A study published in 1990 has stated that ionizing radiation does not increase the risk of pancreatic cancer[9]. These findings are based predominantly on research in people who survived an atomic bomb explosion. The notion that the pancreas is relatively non-sensitive to ionizing radiation has been partially revised by a study of the increased incidence of pancreatic cancer among employees of nuclear research centers in the United States and other countries[12]. Nonetheless, this report[9] has stressed the significance of other concurrent risk factors such as smoking, diabetes, positive family history, or any other pancreatic disease.

Age and race

Age and race are the most prominent confounding factors of pancreatic cancer risk. During the first three decades, pancreatic cancer is a rarity, but from the age of 30 years onwards, its incidence increases significantly, peaking in the seventh to eighth decades, when 80% of adenocarcinomas are diagnosed. The mean age at diagnosis is 65

years. Pancreatic cancer is diagnosed before the age of 50 years only in 10% of patients[13].

With regard to racial differences, pancreatic cancer demonstrates the highest incidence in Afro-Americans in the United States, inhabitants of Northern Europe, in Polynesians in Hawaii, and Maoris in New Zealand. In the United States, the mortality of the Afro-American population is 1.4 times higher than that of the Caucasian population. This fact may be explained by a higher proportion of smokers and patients with diabetes, and at the same time, a positive family history of pancreatic cancer[14].

Hormonal factors and allergy

Pancreatic cancer demonstrates a different incidence in men and women. The cumulative risk in men is 0.2% and 0.1% in women. The lower incidence of pancreatic cancer in women may point to a link between hormonal factors and the development of cancer. The results of studies dealing with the relationship between female hormones and the development of cancer conducted to date have been inconclusive. As studies conducted to date confirm, this malignancy demonstrates minimal estrogen dependency. Parity and duration of hormonal exposure are negatively associated with the degree of risk of pancreatic cancer[15,16]. In the case of postmenopausal women, this risk is not influenced by hormonal substitution. In a number of studies, pancreatic cancer is associated with a higher number of deliveries, with earlier menarche and late menopause, higher age during the first delivery, or hormonal contraception. On the other hand, there exist studies in which the aforementioned factors were associated with a decrease in the risk of pancreatic cancer. An analysis of 10 case-control studies and five cohort studies did not demonstrate any link between hormonal factors and pancreatic cancer in women[17]. Indeed, additional factors, such as differences in life habits, may also contribute to the different cumulative risks between men and women.

Studies published to date have demonstrated a decrease in the risk of pancreatic cancer in individuals with allergies, especially respiratory allergy[18]. Longer survival has been described in allergic patients who have undergone resection procedures compared to resected patients with no allergies. The decrease in risk is most often associated with respiratory forms of allergy such as hay fever, and allergy to pollen and grass. The mechanisms of the possible protective effect of allergy in patients with a tumor is not exactly known[19].

CHRONIC PANCREATITIS

At present, acute and chronic pancreatitis are considered two pathogenically different disease entities; their relation to the genesis of pancreatic cancer is likewise quite different. Although acute pancreatitis is not considered a risk factor in terms of the index diagnosis, the concept of a causal association between alcoholic, hypercalcemic, trophic and hereditary forms of chronic pancreatitis and increased predisposition to developing pancreatic cancer is generally recognized. The obstructive type of chronic pancreatitis has been questioned as a risk factor by some authors[20].

Although no more than 5% of diagnosed cases of pancreatic cancer can be explained by recurrent attacks of chronic pancreatitis, the same genetic changes have been detected in individuals with chronic inflammation of the pancreas and pancreatic cancer. Chronic inflammation is thought to induce genetic alterations in tissue, while the ongoing healing process exposes defective cells to growth factors; the result is a pathological microenvironment in which stromal elements facilitate the neoplastic process in epithelial cells (the so-called feeder theory)[21]. According to this theory, the effector cells of the inflammatory response include active macrophages whose products, particularly the cytokines tumor-necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factor (TGF) α and β induce cell proliferation, angiogenesis and desmoplastic reaction, that is, processes that form part of the pathogenesis of chronic pancreatitis and pancreatic cancer. IL-6 promotes the maturation of myeloid precursors into macrophages, and TGF-α inhibits processes leading to apoptosis and stimulates progressive fibrosis. Additionally, the same cytokine activates the transcription factor nuclear factor (NF)-κB, a mediator of inhibition of programmed cell death. Its expression has been reported in cases of chronic inflammation of the pancreas and pancreatic cancer. In addition, NF-κB upregulation promotes the production of reactive nitrogen oxide and cyclooxygenase (COX)-2 and induces IL-8 expression. There have been reports of an autocrine growth promoting effect of IL-8, which is produced at increased rates in response to hypoxia; particularly in the center of tumor foci, thus having a pro-carcinogenic and pro-metastatic effect. COX-2 responds to increased prostaglandin production in cases of inflammation and cancer, facilitates cell proliferation and angiogenesis, and is a potent inhibitor of apoptosis. Additionally, COX-2 is involved in the transformation of chemical carcinogens into their mutagenic derivatives. This particular ability of COX-2 increases the risk of pancreatic cancer in smokers. Immunohistochemical investigations have shown that COX-2 expression is bound to pancreatic islet Langerhan's cells; as a result, increased expression of the enzyme heralds islet inflammation.

One of the hypotheses of pancreatic cancer development is based on the key relations between islet of Langerhan's inflammation, insulin resistance, growth promotion, and diabetes. Additional products of activated macrophages and neutrophil granulocytes in pancreatitis and pancreatic cancer include reactive forms of oxygen and nitric oxide, whose presence is causally related to DNA structural defects[22]. The risk for developing a malignancy in individuals with chronic pancreatitis is 16 times that of the healthy population. One study has reported in patients with chronic pancreatitis, an increased incidence of both extrapancreatic tumors (relative risk, 1.5) and pancreatic malignancy (relative risk, 18.5). When one considers relevant only conditions that develop during 4 years of chronic inflammation, then the relative

risk increased by a factor of 15.6 for smokers, whereas there was no increase in non-smokers[23]. A prospective study of the French Cancer Registry, including 85% of cases of chronic pancreatitis of alcoholic etiology, has reported a relative risk of 19.0[24]. In a Czech study of 213 patients, 11 of whom had cancer, the prevalence of pancreatic cancer was 5.1%. The interval from establishing the diagnoses of chronic pancreatitis and pancreatic cancer was 6-13 years. The cumulative risk for malignancy in patients with chronic pancreatitis was shown to increase in a linear manner, and was 1.8 and as high as 4.0 after 10 and 20 years, respectively[25]. The priority of current clinical research is to identify patients with sporadic chronic pancreatitis who are at increased risk of developing pancreatic cancer.

GENETIC SUSCEPTIBILITY

Family history of pancreatic cancer

Familial pancreatic cancer (FPC) is defined as two or more first-degree relatives with pancreatic cancer. As an independent nosological unit, FPC represents only 3%-10% of the total number of pancreatic cancers. Nonetheless, the relative risk in such cases is 4.6-32 times higher, depending on the number of afflicted persons within the family[26]. Only 20% of FPC patients demonstrate a genetic abnormality. Nonetheless, individuals from families with FPC should undergo genetic testing for the presence of hereditary breast and ovarian syndrome (BRCA1, BRCA2). These mutations are most often identified in FPC. The relative risk of pancreatic cancer in carriers of the BRCA1 mutation increases to 2.26 and in the case of the BRCA2 mutation to 3.5-8[27].

Pancreatic cancer forms a component of a whole range of hereditary diseases and syndromes.

Cystic fibrosis is an autosomal recessive disease that is caused by mutations in the *CFTR* gene, and is characterized by the production of viscous mucus, which apart from blocking the airways, also leads to obstruction of the pancreatic duct, which increases the risk of inflammation. Patients with CF are at increased risk of chronic pancreatitis and of pancreatic tumors[28].

Familial atypical multiple mole melanoma (FAMMM) is an autosomal dominant disease that is characterized by the occurrence of > 50 atypical nevi and malignant melanoma in two or more first or second-degree relatives. Approximately 10% of melanomas have a familial incidence and the mutation of the *CDKN2A* gene is identified in -40% of these families[29].

Peutz-Jeghers syndrome is an autosomally dominant hereditary disease with characteristic hamartoma polyps of the gastrointestinal tract, and mucocutaneous melanin pigmentation. Almost half of these patients are carriers of a germinal *STK11/LKB1* gene mutation. Thus, afflicted individuals have a 36% risk (cumulative lifetime risk) of developing pancreatic cancer[30].

Hereditary non-polyposis colorectal carcinoma syndrome (HNPCC) is another hereditary cancer syndrome, for which the incidence of pancreatic cancer is typical. This syndrome is caused by mutations in mismatch repair (MMR) genes *MSH2*, *MLH1*, *MSH6* and *PMS2*. The average risk in carriers of MMR gene mutations is 5%-10%. Pancreatic cancer is approximately seven times more frequent in carriers of *MMR* gene mutations, and in these individuals, it is 15 times more often diagnosed before the age of 60 years[31]. Apart from the aforementioned, pancreatic cancer can occur in association with other diseases such as Li-Fraumeni syndrome, ataxia-teleangiectasia syndrome, multiple endocrine neoplasia type I syndrome (MENI) or Von Hippel-Lindau syndrome.

Hereditary pancreatitis

Hereditary pancreatitis is currently considered to be an independent nosological unit. This is an autosomally dominant disease with 80% penetrance. In patients with hereditary pancreatitis, trypsin becomes activated while still in the pancreas. This accounts for partial digestion of the pancreatic tissue, which causes irritation and inflammation. A strong genetic association exists with mutations found in the *PRSS1*, *SPINK1* and *CFTR* genes[32]. Patients with this hereditary pancreatitis have a 40-60-fold higher risk of developing pancreatic cancer. If such predisposed individuals are smokers, then the development of pancreatic cancer, or rather its diagnosis, shifts to younger age categories, in which it occurs up to two decades earlier than in non-smokers. Similarly, alcohol consumption also leads to earlier diagnosis of cancer, also 20 years earlier[2].

DIABETES MELLITUS

A mutual association between pancreatic cancer and diabetes mellitus has long been monitored. However, the issue of mutual linkage is complicated by the fact that, while long-term diabetes is considered to be an etiological factor of the cancer, newly developed diabetes is an early manifestation of the cancer[33].

The pathogenesis of diabetes associated with cancer and the biochemical mediators involved have not been completely elucidated. Its development due to the mere destruction of pancreatic tissue by the tumor or as a consequence of chronic pancreatitis is less probable. The high prevalence of diabetes and disorders of glucose tolerance in small, early carcinomas (< 20 mm), and primary detection of diabetes nearly 2 years before the diagnosis of carcinoma, points to the influence of humoral markers rather than to local effects of the tumor. Further research is necessary to clarify the pathogenesis of carcinoma-associated diabetes, and to uncover new markers that can differentiate it from type 2 diabetes[33]. Newly developed diabetes during a period of < 2 years prior to the diagnosis of carcinoma is a promising sign of the presence of a completely asymptomatic carcinoma. This is why screening of sporadic, early pancreatic cancer in persons with newly diagnosed diabetes is being considered. The interval between primary detection of diabetes and the diagnosis of carcinoma ranges between 5 and 29 mo[34].

Primary detection of hyperglycemia and diabetes represent a reference point for the timely diagnosis of sporadic pancreatic carcinoma before symptoms develop. It is well known that the symptoms of pancreatic cancer occur only weeks or months before diagnosis, which usually means that an advanced, non-resectable tumor is present and expected survival is only 4-6 mo. If we monitor glycemia in patients with small, resectable carcinomas (< 20 mm), then most suffer from disorders of glucose tolerance. Studies have demonstrated in 55%-65% of patients with resectable carcinoma, a disorder of glucose tolerance or newly developed diabetes during a period of < 2 years prior to the diagnosis of carcinoma[34,35]. Approximately half of patients with sporadic carcinoma suffer from diabetes, and in almost 50%, diabetes is diagnosed at the time of carcinoma diagnosis. It is highly probable that diabetes precedes the diagnosis of the malignancy by several months or even years. The aforementioned facts indicate the application of pancreatic cancer screening in asymptomatic individuals with newly diagnosed diabetes[33].

Studies conducted to date have shown that the prevalence of diabetes (determined on the basis of the oral glucose test, fasting blood glucose, and meeting American Diabetes Association criteria) in patients with pancreatic cancer is 45%-65%. In the original study, diabetes was newly diagnosed concurrently with carcinoma in 40% of cases[34]. In other studies, the percentage of newly diagnosed diabetes was as high as 74%-88%. In summary, it could be concluded that the majority of diabetes associated with pancreatic cancer represents *de novo* diabetes, that is, diagnosed during a period of 2 years preceding pancreatic cancer, and almost half of patients with early carcinoma have diabetes. Moreover, diabetes that develops in this way usually improves following pancreatic resection.

MOLECULAR MECHANISMS

Model of pancreatic neoplasia

Molecular mechanisms of solid cancer are very complex with different mechanisms taking place and affecting the tissue at different stages of the disease. Detailed molecular mechanisms of initiation, development and progression of pancreatic cancer have been thoroughly studied since the basic principles of the disease were revealed in the 1970s and 1980s[36-40]. The classic model of pancreatic cancer development describes morphological as well as molecular transformation from precursor lesions into invasive carcinoma[41]. The standard nomenclature and diagnostic criteria for classification of duct lesions has primarily been based on grades of pancreatic intraepithelial neoplasia (PanIN)[42]. The grades 1A, 1B, 2 and 3 represent growing cytological atypia characterized by loss of polarity, nuclear crowding, enlarged nuclei, pseudo-stratification and hyperchromatism. Each PanIN stage is characterized by a distinct pattern of molecular processes that are characterized by genetic irregularities that affect specific genes and genetic pathways.

Proto-oncogenes and tumor suppressors

In the PanIN model, genetic alterations have a fundamental role affecting key guardians of cellular signaling, which induces instability of entire molecular systems such as cell growth, division, apoptosis and migration. Proto-oncogenes code for proteins that act as positive regulators for these systems, such as growth factors, signal transducers, transcription factors or apoptotic inhibitors. Their mutated forms, oncogenes, are often present in cancer cells. The mutation causes the protein products of oncogenes to be permanently activated, which results in uncontrolled cell proliferation. Oncogenic mutations have a dominant character; therefore, deficiency of one allele (i.e. heterozygous mutation) is sufficient for a fatal outcome. There are several key proto-oncogenes involved in pancreatic cancerogenesis, including KRAS, CTNNB1 (β-catenin), PIK3CA or AKT1. The most common oncogenic mutation types are point mutation, deletion, gene amplification, and gene rearrangement.

Tumor suppressor genes code for proteins that act against cell proliferation, such as signaling inhibitors, negative transcription factors, activators of apoptosis, or members of DNA repair systems. As a result of genetic alteration, their normal function may be reduced or eliminated completely. Mutations in tumor suppressor genes have a recessive character; hence, the cell looses their function only when both alleles are affected. In the most common case, described as a double hit model, one allele is initially mutated while the other is subsequently mutated or lost completely[43]. A separate mechanism of tumor suppressor deactivation is by hypermethylation[44]. In pancreatic cancer, the frequently affected tumor suppressors include TP53, APC, SMAD4 and TP16. The genes most frequently mutated in pancreatic cancer are listed in Table 2[45,46].

Signaling pathways in pancreatic cancer c-MET/HGF signaling pathway

The c-MET/HGF (hepatocyte growth factor) signaling pathway is a key factor in early progression of pancreatic cancer. The pathway is responsible for invasive growth through activation of key oncogenes, angiogenesis and scattering (cell dissociation and metastasis). c-MET is a proto-oncogene that encodes an HGF receptor that has a primary function in embryonic development and wound healing[47]. Although c-MET mRNA is present at very low levels in normal human exocrine pancreas, it is upregulated in a majority of pancreatic cancers[48-50], as well as in pancreatitis-affected epithelial cells[51]. Overexpression of c-MET is also observed in regenerating tissue affected by acute pancreatitis[52], and it is seen as an early event in pancreatic cancerogenesis[51]. HGF is a primary ligand of c-MET. Upon c-MET/HGF interaction, several different signaling pathways are activated, including the Ras, phosphoinositide 3-kinase (PI3K), Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and β-catenin (Wnt) pathways.

Ras/Raf/MAPK pathway

The Ras/Raf/mitogen-activated protein kinase (MAPK) pathway is one of the most studied and best described signaling pathways in cancer[53]. The role of Ras/Raf/

Table 2 Genes most frequently mutated in pancreatic cancer[45,46]

Gene symbols	Protein name	Mutation frequency (%)	Type	Main signaling or system
KRAS	K-ras	58	Proto-oncogene	*Ras/Raf/MAPK*
TP53	Tumor protein p53	37	Tumor suppressor	*Apoptosis Cell cycle control*
CDKN2A	Tumor protein p16 (INK4A)	29	Tumor suppressor	*Cell cycle control*
CTNNB1	β-catenin	24	Proto-oncogene	*Wnt*
SMAD4 DPC4	Smad Dpc4	22	Tumor suppressor	*TNFbeta/SMAD*
APC	Apc	16	Tumor suppressor	*Wnt*
PIK3CA	Phosphoinositide 3-kinase	5	Proto-oncogene	*PTEN/PI3K/AKT*

DPC: Deleted in pancreatic cancer; APC: Adenomatous polyposis coli; MAPK: Mitogen-activated protein kinase.

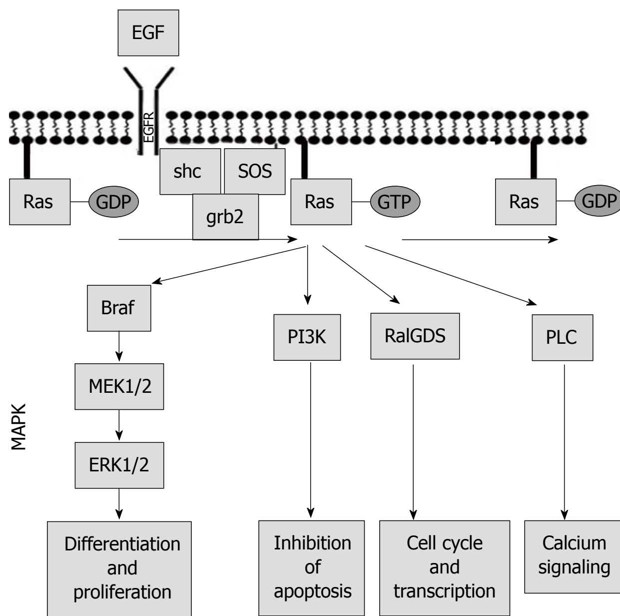


Figure 2 Ras/Raf/mitogen-activated protein kinase pathway.

MAPK signaling is critical for many cancerogenic processes, including cell growth and division, cell differentiation and migration, tissue healing and repair, and angiogenesis. The central regulator of the signal transduction from extracellular to intracellular environment is Ras protein, which is localized at the inner wall of the cellular membrane. Under normal physiological conditions, the hydrophobic Ras protein is inactive and bound to GDP. An extracellular signal coming through growth factor transmembrane receptors (such as growth factor receptors or cytokine receptors) promotes release of a guanidine exchange factor SOS, which initiates removal of GDP from Ras protein and its subsequent activation upon binding to GTP. Activated GTP-Ras complex triggers a kinase activity of Raf kinase, which ultimately results in activation of an MAPK, an important regulator of DNA transcription and mRNA translation. Mutations that affect any of the Ras/Raf/MAPK members produce an increase in tumorigenicity. Aside from Raf and MAPK, there are other downstream effectors of Ras protein, including PI3K, thus providing crosstalk between multiple pathways (Figure 2).

PTEN/PI3K/AKT pathway

PTEN/PI3K/AKT is a significant signaling pathway that is fundamentally based on regulated activation of AKT oncogene through its localization at the cell membrane[54]. The two important protein families involved in the membrane localization of AKT are PI3K and PTEN phosphatases.

PI3K is able to phosphorylate certain membrane-bound lipids known as phosphoinositides. The PI3K-mediated phosphorylation may progress in three stages, which produces phosphatidylinositol 3-phosphate (PIP), phosphatidylinositol (3,4)-bisphosphate (PIP_2), and phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). The phosphorylated forms, PIP_3 and, to a lesser extent, PIP_2, attract important protein kinases to the cell membrane. The most prominent is AKT, a family of serine/threonine protein kinases that trigger a number of key cellular processes, including glucose metabolism, cell proliferation, apoptosis, transcription, and cell migration. AKT activity is strongly dependent on its proper localization on the cell membrane. The positioning of AKT at the membrane is achieved through its strong binding to PIP_3. In pancreatic carcinogenesis, AKT1 acts as an oncogene that upholds cell survival by overcoming cell cycle arrest[55-57], blocking apoptosis[58-60], and promoting angiogenesis[61]. PTEN is a phosphatase that acts in opposition to PI3K. It has tumor suppression ability by converting PIP_3 back to PIP_2 and to PIP, hence disrupting membrane localization and reducing activity of AKT[62]. In most cancers, expression levels of PI3Ks and AKT are high, while PTEN is often deactivated by mutation, or deleted completely. Through its key role in pancreatic carcinogenesis, PI3K/AKT/PTEN signaling is an important target for anticancer therapy.

JAK/STAT pathway

The JAK/STAT signaling pathway has an important role in regulation of DNA transcription by transmission of chemical signals from cytokine receptors into the cell nucleus. The signal is passed upon phosphorylation of receptor tyrosine residues by JAK prompting activation and dimerization in a family of STAT proteins. Activated STAT dimers initiate DNA transcription inside the nucleus. It is known that inhibition of JAK/STAT signaling induces apoptosis in various human cancers, and is there-

fore, a primary focus for potential new drug candidates[63]. A recent study has reported reduced growth of pancreatic cancer cells *in vitro* when exposed to benzyl isothiocyanate (BITC), due to its suppression of STAT3 signaling and subsequent induction of apoptosis. This is suggested as a possible explanation of the anticarcinogenic effect of cruciferous vegetables (such as broccoli, cauliflower, cabbage or horseradish) that are rich in BITC[64].

TGF-β/SMAD signaling

TGF-β is a ligand that binds to type Ⅱ cytokine receptor dimer, which then binds and activates type Ⅰ cytokine receptor dimer, which triggers phosphorylation of receptor-regulated SMADs (R-SMADs), mainly SMAD2 and SMAD3. In phosphorylated form, the R-SMADs form a complex with SMAD4, which accumulates in the nucleus and interacts with other factors to stimulate transcription of genes that are important for cell cycle arrest and migration. SMAD4 is therefore a key mediator for TGF-β signals. Due to its frequent absence in proliferating pancreatic cancer tissue, it is also known as DPC or "deleted in pancreatic cancer"[65]. Relatively high frequency of SMAD4 mutations and loss of heterozygosity at the DPC4 locus (18q21.1) strongly suggest that the protein is a primary tumor suppressor that is involved in pancreatic cancerogenesis. However, reinstating SMAD4 expression results in tumor growth suppression only *in vivo* and not *in vitro*. It has also been found that SMAD4-independent pathways may be responsible for tumorigenic effect of TGF-β signaling[66].

Wnt signaling

Wnt signaling is crucial to formation and maintenance of endocrine pancreas[67]. During pancreatic carcinogenesis, Wnt triggers transcription of a number of genes that have a direct impact on cell proliferation, differentiation and migration[68]. Activation of Wnt signaling is by interaction of a family of membrane-bound receptors known as Frizzleds with Wnt ligands. Once activated, the downstream signals may proceed through separate pathways. In a canonical pathway, signal transduction is mediated by stabilization and translocation of β-catenin from the cytosol into the nucleus followed by its interaction with T-cell factor (HMG box) which activates transcription of target genes. The localization of high expression levels of β-catenin at the nucleus has been experimentally confirmed for various high grade PanIN lesions, as well as in advanced pancreatic cancer[69]. In a non-canonical, β-catenin-independent pathway, other signal mediators are involved, which block the β-catenin-assisted transcription. The nuclear localization of β-catenin and high expression levels of WNT5a, a gene involved in non-canonical Wnt pathways, suggests involvement of both pathways in pancreatic cancer progression[68].

CDKN2A and cell cycle control

The cell cycle control genes have profound importance in cancer and CDKN2A is one of key factors in its negative control. The CDKN2A has two promoters and alternative splicing sites that result in two alternative protein products: cyclin-dependent kinase inhibitor p16INK4a and p53-activator p14ARF. Although both proteins are active in negative control of the cell cycle, only the function of p16INK4a is frequently lost in pancreatic tumors due to point mutations, deletions or hypermethylation[70]. p16INK4a protein (also known as p16) inhibits key elements of cell cycle progression at the G1 checkpoint. p16 inactivation is an early event in pancreatic carcinogenesis, and low levels of p16 expression are associated with larger tumors, risk of early metastases and poor survival[71].

MOLECULAR DIAGNOSTICS

A whole range of findings regarding the molecular biological basis of malignant transformation in pancreatic cancer has been published in recent years, and certain progress has been achieved also in the diagnosis, staging and treatment of localized tumors. In the fields of prevention, early diagnosis, screening and treatment of advanced tumors, which represent the majority of newly diagnosed cases, research has failed to provide any fundamental discoveries that would significantly affect the prognosis of patients with pancreatic cancer. Better understanding of the mechanisms of molecular genetics involved in pancreatic carcinogenesis has enabled the identification of a number of hereditary syndromes that in probands represent an increased risk of cancer. An overview is shown in Table 3[72].

Carbohydrate antigen (Ca) 19-9 retains its dominant role among tumor markers. It is the only marker to have been applied in clinical practice, where it is used to detect early recurrence in patients with an already known diagnosis and those undergoing treatment[73]. Use of Ca 19-9 as a screening test has yielded unsatisfactory results. This marker is not specific for pancreatic cancer and may be elevated in various cholestatic syndromes, and not necessarily a tumor. Moreover, the levels of secreted Ca 19-9 are affected by positivity of the Lewis antigen a and b[74]. Table 4 presents an overview of other biomarkers being studied as potentially useful in the diagnosis of pancreatic cancer[75].

Currently, none of the listed biomarkers meets the criteria of utility for the detection of early carcinoma. Even the promising marker PAM4 has demonstrated a sensitivity of 54% in early stage 1a carcinoma and 75% in stage 1b carcinoma[75]. The common denominator of the failure of all biomarkers in early detection lies in their low sensitivity; in some cases, associated with difficult or an invasive collection of biological material.

Detection of early pancreatic cancer in the general population using currently available means is impossible. Interest is now focusing on at-risk groups; especially those in whom the risk of developing this cancer is at least 10-fold higher compared to the general population. This risk may be stratified into low, intermediate and high[76]. Table 5 summarizes this risk stratification.

Several studies published recently have attempted to detect early and resectable carcinoma in high-risk groups with the aid of imaging methods[72,76]. Screening exami-

Table 3 Overview of hereditary syndromes predisposing to pancreatic cancer

Syndrome	Gene	Life-time risk	Relative risk
FAMMM	CDKN2A	10%-15%	20-34
HBOC	BRCA2	5%	10
HBOC	BRCA1	Not known	2
Hereditary pancreatitis	PRSS1/TRY1	30%-50%	50
Lynch syndrome	MLH1/MSH2	Not known	Not known
Peutz-Jeghers syndrome	STK11/LKB1	36%	136
FAP	APC	Not known	4
Li-Faumeni syndrome	p53	Not known	Not known
FPC	Not known	Up to 50%	18-57

APC: Adenomatous polyposis coli; FAMMM: Familial atypical multiple mole melanoma; FAP: Familial adenomatous polyposis; FPC: Familial pancreatic cancer; HBOC: Hereditary breast and ovarian syndrome. Adapted from[72].

Table 4 Overview of biomarkers in pancreatic cancer

Biomarker	Sensitivity (%)	Specificity (%)
CEA	45	75
Carcinoembryonic antigen-related cell adhesion molecule-1	85	98
Ca 19-9	80	73
SPan-1	81-94	75
DUPAN-2	48-80	75-85
Macrophage inhibitory cytokine 1	90	62
Alpha4GnT	76	83
PAM4	77	95
Pancreatic juice DNA methylation	82	100
Fecal K-ras	77	81

CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; DUPAN-2: Pancreatic cancer-associated antigen 2; PAM4: Peptidylglycine alpha-amidating monooxygenase 4.

Table 5 Level of risk according to cumulative risk factors

Factors	Risk level
Race/sex: Male; black; Ashkenazi Jewish descent. Exposures: obesity; smoking; diabetes mellitus; *Helicobacter pylori* infection. Family history: Cancer history in a first-degree relative; history of pancreatic cancer in one first-degree relative. Inherited conditions: Hereditary non-polyposis colorectal cancer; familial adenomatous polyposis; *BRCA1* mutation carrier	Low (less than 5-fold)
Family history: History of pancreatic cancer in two first-degree relatives. Inherited conditions: cystic fibrosis; *BRCA2* mutation carrier. Comorbidity: Chronic pancreatitis	Moderate (5- to 10-fold)
Inherited conditions: FAMMM kindreds with p16 germline mutation and at least one case of pancreatic cancer in a first-degree or second-degree relative; hereditary pancreatitis; Peutz-Jeghers syndrome; *BRCA2* or *BRCA1* mutation carrier with at least one case of pancreatic cancer in a first-degree or second-degree relative. Family history: Three or more first-degree; second-degree or third-degree relatives with pancreatic cancer	High (greater than 10-fold)

FAMMM: Familial atypical multiple mole melanoma.

nations usually include some type of imaging method, such as endoscopic ultrasound, magnetic resonance imaging, computer tomography, or genetic tests. Successful identification of small tumors or precursor lesions, cystic tumors and intraductal papillary mucinous neoplasia is now possible in the at-risk population to a higher degree than in the general population. In cases in which solid ductal adenocarcinoma is uncovered, these lesions are usually resectable. Nonetheless, according to the available literature, recurrence has been demonstrated in all such patients over a period of several months.

In recent years, clinical research has focused on identifying patients with chronic pancreatitis with a high risk of developing pancreatic cancer. As discussed above, the following risk factors have been identified to date: smoking[23], duration of chronic pancreatitis[25], status after surgery for chronic pancreatitis in symptomatic individuals (recurrent pain, jaundice, weight loss, loss of appetite)[77], presence of a mutated form of the K-ras oncogene in a sample obtained using fine-needle aspiration biopsy or pancreatic duct brush cytology[78], loss of suppressor gene p16 expression[79], and polymorphism of the uridine diphosphate glucuronyltransferase gene (presence of the UGT1A7 allele causing low detoxification activity of the enzyme)[80].

In view of the completely different pathogenesis of acute and chronic pancreatitis and thus the different relationship to the development of pancreatic cancer, acute pancreatitis currently is not considered to be a risk factor for the development of pancreatic cancer. On the contrary, the association between alcoholic, hypercalcemic, tropical and hereditary chronic pancreatitis and the increased risk of pancreatic cancer is generally valid. The risk of developing pancreatic cancer in patients with chronic pancreatitis is up to 16-fold higher compared to the healthy population. K-ras mutations are detectable in nearly 80% of patients with carcinoma. Detection of K-ras mutations in patients with chronic pancreatitis may thus be used in combination with other methods as a screening test for the detection of early carcinoma[81].

As described above, much attention is also being paid currently to the relationship between newly diagnosed type 2 diabetes mellitus and pancreatic cancer, whereby diabetes is considered to be an early manifestation of pancreatic cancer, preceding the usual clinical manifestations of this malignancy. Patients with newly diagnosed diabetes will probably be considered to be at higher risk than they are today and will be screened. However, patients with diabetes who are suitable for screening will need to undergo multilevel selection, and the diagnosis of diabetes itself will represent the first filter of such a process. The second level should be the presence of one of the current biomarkers, or preferably the identification of a new marker with a higher predictive value. No

such marker is currently available, therefore, predictive computer models are also being envisaged.

Recent studies have demonstrated in pancreatogenic diabetes mellitus a protective effect of metformin for decreasing the risk of developing pancreatic cancer in patients with chronic pancreatitis. In contrast, treatment with insulin or its secretagogues increases the risk of carcinoma in these patients[82].

CONCLUSION

Uncovering the importance of basic risk factors such as chronic pancreatitis and diabetes mellitus, along with a detailed knowledge of fundamental molecular processes is expected to assist in reducing mortality of pancreatic cancer through development of new approaches for detection of early stages of the disease. This will mainly be applied to evaluation of the survival prognosis and rational selection of therapy; most importantly, with respect to options for radical surgical treatment. In addition, identification of specific genetic aberrations may serve as key molecular markers as predictors of response for targeted therapies. The response prediction should not only prolong survival, but also improve the quality of life for most advanced stages of the disease.

ACKNOWLEDGMENTS

Authors would like to thank Dr. Lucie Benesova for her assistance with detailed description of signaling pathways and Professor Marcela Kopacova for helpful discussions and comments on the manuscript.

REFERENCES

1 **Lowenfels AB**, Maisonneuve P. Epidemiology and risk factors for pancreatic cancer. *Best Pract Res Clin Gastroenterol* 2006; **20**: 197-209
2 **Chu D**, Kohlmann W, Adler DG. Identification and screening of individuals at increased risk for pancreatic cancer with emphasis on known environmental and genetic factors and hereditary syndromes. *JOP* 2010; **11**: 203-212
3 **Dusek L**, Muzík J, Gelnarová E, Fínek J, Vyzula R, Abrahámová J. Cancer incidence and mortality in the Czech Republic. *Klin Onkol* 2010; **23**: 311-324
4 **Genkinger JM**, Spiegelman D, Anderson KE, Bergkvist L, Bernstein L, van den Brandt PA, English DR, Freudenheim JL, Fuchs CS, Giles GG, Giovannucci E, Hankinson SE, Horn-Ross PL, Leitzmann M, Männistö S, Marshall JR, McCullough ML, Miller AB, Reding DJ, Robien K, Rohan TE, Schatzkin A, Stevens VL, Stolzenberg-Solomon RZ, Verhage BA, Wolk A, Ziegler RG, Smith-Warner SA. Alcohol intake and pancreatic cancer risk: a pooled analysis of fourteen cohort studies. *Cancer Epidemiol Biomarkers Prev* 2009; **18**: 765-776
5 **Decker GA**, Batheja MJ, Collins JM, Silva AC, Mekeel KL, Moss AA, Nguyen CC, Lake DF, Miller LJ. Risk factors for pancreatic adenocarcinoma and prospects for screening. *Gastroenterol Hepatol* (N Y) 2010; **6**: 246-254
6 **Harnack LJ**, Anderson KE, Zheng W, Folsom AR, Sellers TA, Kushi LH. Smoking, alcohol, coffee, and tea intake and incidence of cancer of the exocrine pancreas: the Iowa Women's Health Study. *Cancer Epidemiol Biomarkers Prev* 1997; **6**: 1081-1086
7 **Calle EE**, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003; **348**: 1625-1638
8 **Nkondjock A**, Krewski D, Johnson KC, Ghadirian P. Dietary patterns and risk of pancreatic cancer. *Int J Cancer* 2005; **114**: 817-823
9 BEIR V: implications for the nuclear workforce. *Science* 1990; **247**: 620-622
10 **Nöthlings U**, Wilkens LR, Murphy SP, Hankin JH, Henderson BE, Kolonel LN. Meat and fat intake as risk factors for pancreatic cancer: the multiethnic cohort study. *J Natl Cancer Inst* 2005; **97**: 1458-1465
11 **Ojajärvi A**, Partanen T, Ahlbom A, Boffetta P, Hakulinen T, Jourenkova N, Kauppinen T, Kogevinas M, Vainio H, Weiderpass E, Wesseling C. Risk of pancreatic cancer in workers exposed to chlorinated hydrocarbon solvents and related compounds: a meta-analysis. *Am J Epidemiol* 2001; **153**: 841-850
12 **Tilyou SM**. BEIR V report. Experts urge cautious interpretation of higher risk estimates. *J Nucl Med* 1990; **31**: 13A-19A
13 **Li D**, Morris JS, Liu J, Hassan MM, Day RS, Bondy ML, Abbruzzese JL. Body mass index and risk, age of onset, and survival in patients with pancreatic cancer. *JAMA* 2009; **301**: 2553-2562
14 **Silverman DT**, Hoover RN, Brown LM, Swanson GM, Schiffman M, Greenberg RS, Hayes RB, Lillemoe KD, Schoenberg JB, Schwartz AG, Liff J, Pottern LM, Fraumeni JF Jr. Why do Black Americans have a higher risk of pancreatic cancer than White Americans? *Epidemiology* 2003; **14**: 45-54
15 **Kreiger N**, Lacroix J, Sloan M. Hormonal factors and pancreatic cancer in women. *Ann Epidemiol* 2001; **11**: 563-567
16 **Skinner HG**, Michaud DS, Colditz GA, Giovannucci EL, Stampfer MJ, Willett WC, Fuchs CS. Parity, reproductive factors, and the risk of pancreatic cancer in women. *Cancer Epidemiol Biomarkers Prev* 2003; **12**: 433-438
17 **Wahi MM**, Shah N, Schrock CE, Rosemurgy AS 2nd, Goldin SB. Reproductive factors and risk of pancreatic cancer in women: a review of the literature. *Ann Epidemiol* 2009; **19**: 103-111
18 **Eppel A**, Cotterchio M, Gallinger S. Allergies are associated with reduced pancreas cancer risk: A population-based case-control study in Ontario, Canada. *Int J Cancer* 2007; **121**: 2241-2245
19 **Olson SH**, Chou JF, Ludwig E, O'Reilly E, Allen PJ, Jarnagin WR, Bayuga S, Simon J, Gonen M, Reisacher WR, Kurtz RC. Allergies, obesity, other risk factors and survival from pancreatic cancer. *Int J Cancer* 2010; **127**: 2412-2419
20 **Cavestro GM**, Comparato G, Nouvenne A, Sianesi M, Di Mario F. The race from chronic pancreatitis to pancreatic cancer. *JOP* 2003; **4**: 165-168
21 **Farrow B**, Evers BM. Inflammation and the development of pancreatic cancer. *Surg Oncol* 2002; **10**: 153-169
22 **Lowenfels AB**, Maisonneuve P, Lankisch PG. Chronic pancreatitis and other risk factors for pancreatic cancer. *Gastroenterol Clin North Am* 1999; **28**: 673-685, x
23 **Talamini G**, Falconi M, Bassi C, Sartori N, Salvia R, Caldiron E, Frulloni L, Di Francesco V, Vaona B, Bovo P, Vantini I, Pederzoli P, Cavallini G. Incidence of cancer in the course of chronic pancreatitis. *Am J Gastroenterol* 1999; **94**: 1253-1260
24 **Malka D**, Hammel P, Maire F, Rufat P, Madeira I, Pessione F, Lévy P, Ruszniewski P. Risk of pancreatic adenocarcinoma in chronic pancreatitis. *Gut* 2002; **51**: 849-852
25 **Dítě P**, Pazourková M, Růzicka M, Precechtělová M, Novotný I, Dastych M. [Chronic pancreatitis as a risk factor for pancreatic carcinoma]. *Vnitr Lek* 2002; **48**: 638-641
26 **Lynch HT**, Lanspa SJ, Fitzgibbons RJ Jr, Smyrk T, Fitzsimmons ML, McClellan J. Familial pancreatic cancer (Part 1): Genetic pathology review. *Nebr Med J* 1989; **74**: 109-112
27 **Ferrone CR**, Levine DA, Tang LH, Allen PJ, Jarnagin W, Brennan MF, Offit K, Robson ME. BRCA germline muta-

tions in Jewish patients with pancreatic adenocarcinoma. *J Clin Oncol* 2009; **27**: 433-438
28 **Neglia JP**, FitzSimmons SC, Maisonneuve P, Schöni MH, Schöni-Affolter F, Corey M, Lowenfels AB. The risk of cancer among patients with cystic fibrosis. Cystic Fibrosis and Cancer Study Group. *N Engl J Med* 1995; **332**: 494-499
29 **Lynch HT**, Fusaro RM, Lynch JF, Brand R. Pancreatic cancer and the FAMMM syndrome. *Fam Cancer* 2008; **7**: 103-112
30 **Latchford A**, Greenhalf W, Vitone LJ, Neoptolemos JP, Lancaster GA, Phillips RK. Peutz-Jeghers syndrome and screening for pancreatic cancer. *Br J Surg* 2006; **93**: 1446-1455
31 **Yamamoto H**, Itoh F, Nakamura H, Fukushima H, Sasaki S, Perucho M, Imai K. Genetic and clinical features of human pancreatic ductal adenocarcinomas with widespread microsatellite instability. *Cancer Res* 2001; **61**: 3139-3144
32 **Keiles S**, Kammesheidt A. Identification of CFTR, PRSS1, and SPINK1 mutations in 381 patients with pancreatitis. *Pancreas* 2006; **33**: 221-227
33 **Pannala R**, Basu A, Petersen GM, Chari ST. New-onset diabetes: a potential clue to the early diagnosis of pancreatic cancer. *Lancet Oncol* 2009; **10**: 88-95
34 **Pelaez-Luna M**, Takahashi N, Fletcher JG, Chari ST. Resectability of presymptomatic pancreatic cancer and its relationship to onset of diabetes: a retrospective review of CT scans and fasting glucose values prior to diagnosis. *Am J Gastroenterol* 2007; **102**: 2157-2163
35 **Chari ST**. Detecting early pancreatic cancer: problems and prospects. *Semin Oncol* 2007; **34**: 284-294
36 **Pour P**, Althoff J, Krüger FW, Mohr U. Improvement of pancreatic cancer model by modified treatment with N-nitroso-bis (2-oxopropyl) amine. *Cancer Lett* 1977; **2**: 233-237
37 **Sayers HJ**, Orloff MJ. Development of an animal model of pancreatic cancer. *Surg Forum* 1976; **27**: 456-458
38 **Morosco GJ**, Goeringer GC. Lifestyle factors and cancer of the pancreas: a hypothetical mechanism. *Med Hypotheses* 1980; **6**: 971-985
39 **Lin RS**, Kessler II. A multifactorial model for pancreatic cancer in man. Epidemiologic evidence. *JAMA* 1981; **245**: 147-152
40 **Berlin NI**, Williams M. Pancreatic cancer: an epidemiologic approach and model. *JAMA* 1981; **245**: 171
41 **Hruban RH**, Goggins M, Parsons J, Kern SE. Progression model for pancreatic cancer. *Clin Cancer Res* 2000; **6**: 2969-2972
42 **Hruban RH**, Adsay NV, Albores-Saavedra J, Compton C, Garrett ES, Goodman SN, Kern SE, Klimstra DS, Klöppel G, Longnecker DS, Lüttges J, Offerhaus GJ. Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions. *Am J Surg Pathol* 2001; **25**: 579-586
43 **Serra E**, Puig S, Otero D, Gaona A, Kruyer H, Ars E, Estivill X, Lázaro C. Confirmation of a double-hit model for the NF1 gene in benign neurofibromas. *Am J Hum Genet* 1997; **61**: 512-519
44 **Herman JG**, Jen J, Merlo A, Baylin SB. Hypermethylation-associated inactivation indicates a tumor suppressor role for p15INK4B. *Cancer Res* 1996; **56**: 722-727
45 **Jackson MA**, Lea I, Rashid A, Peddada SD, Dunnick JK. Genetic alterations in cancer knowledge system: analysis of gene mutations in mouse and human liver and lung tumors. *Toxicol Sci* 2006; **90**: 400-418
46 **Bamford S**, Dawson E, Forbes S, Clements J, Pettett R, Dogan A, Flanagan A, Teague J, Futreal PA, Stratton MR, Wooster R. The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *Br J Cancer* 2004; **91**: 355-358
47 **Chmielowiec J**, Borowiak M, Morkel M, Stradal T, Munz B, Werner S, Wehland J, Birchmeier C, Birchmeier W. c-Met is essential for wound healing in the skin. *J Cell Biol* 2007; **177**: 151-162
48 **Ebert M**, Yokoyama M, Friess H, Büchler MW, Korc M. Coexpression of the c-met proto-oncogene and hepatocyte growth factor in human pancreatic cancer. *Cancer Res* 1994; **54**: 5775-5778
49 **Di Renzo MF**, Poulsom R, Olivero M, Comoglio PM, Lemoine NR. Expression of the Met/hepatocyte growth factor receptor in human pancreatic cancer. *Cancer Res* 1995; **55**: 1129-1138
50 **Kiehne K**, Herzig KH, Fölsch UR. c-met expression in pancreatic cancer and effects of hepatocyte growth factor on pancreatic cancer cell growth. *Pancreas* 1997; **15**: 35-40
51 **Yu J**, Ohuchida K, Mizumoto K, Ishikawa N, Ogura Y, Yamada D, Egami T, Fujita H, Ohashi S, Nagai E, Tanaka M. Overexpression of c-met in the early stage of pancreatic carcinogenesis; altered expression is not sufficient for progression from chronic pancreatitis to pancreatic cancer. *World J Gastroenterol* 2006; **12**: 3878-3882
52 **Otte JM**, Kiehne K, Schmitz F, Fölsch UR, Herzig KH. C-met protooncogene expression and its regulation by cytokines in the regenerating pancreas and in pancreatic cancer cells. *Scand J Gastroenterol* 2000; **35**: 90-95
53 **Molina JR**, Adjei AA. The Ras/Raf/MAPK pathway. *J Thorac Oncol* 2006; **1**: 7-9
54 **Carnero A**, Blanco-Aparicio C, Renner O, Link W, Leal JF. The PTEN/PI3K/AKT signalling pathway in cancer, therapeutic implications. *Curr Cancer Drug Targets* 2008; **8**: 187-198
55 **Perugini RA**, McDade TP, Vittimberga FJ Jr, Callery MP. Pancreatic cancer cell proliferation is phosphatidylinositol 3-kinase dependent. *J Surg Res* 2000; **90**: 39-44
56 **Lu X**, Qian J, Yu Y, Yang H, Li J. Expression of the tumor suppressor ARHI inhibits the growth of pancreatic cancer cells by inducing G1 cell cycle arrest. *Oncol Rep* 2009; **22**: 635-640
57 **Roy SK**, Srivastava RK, Shankar S. Inhibition of PI3K/AKT and MAPK/ERK pathways causes activation of FOXO transcription factor, leading to cell cycle arrest and apoptosis in pancreatic cancer. *J Mol Signal* 2010; **5**: 10
58 **Yao Z**, Okabayashi Y, Yutsudo Y, Kitamura T, Ogawa W, Kasuga M. Role of Akt in growth and survival of PANC-1 pancreatic cancer cells. *Pancreas* 2002; **24**: 42-46
59 **Mortenson MM**, Galante JG, Gilad O, Schlieman MG, Virudachalam S, Kung HJ, Bold RJ. BCL-2 functions as an activator of the AKT signaling pathway in pancreatic cancer. *J Cell Biochem* 2007; **102**: 1171-1179
60 **Ripka S**, Neesse A, Riedel J, Bug E, Aigner A, Poulsom R, Fulda S, Neoptolemos J, Greenhalf W, Barth P, Gress TM, Michl P. CUX1: target of Akt signalling and mediator of resistance to apoptosis in pancreatic cancer. *Gut* 2010; **59**: 1101-1110
61 **Ma J**, Sawai H, Ochi N, Matsuo Y, Xu D, Yasuda A, Takahashi H, Wakasugi T, Takeyama H. PTEN regulates angiogenesis through PI3K/Akt/VEGF signaling pathway in human pancreatic cancer cells. *Mol Cell Biochem* 2009; **331**: 161-171
62 **Maitra A**, Hruban RH. A new mouse model of pancreatic cancer: PTEN gets its Akt together. *Cancer Cell* 2005; **8**: 171-172
63 **Buettner R**, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. *Clin Cancer Res* 2002; **8**: 945-954
64 **Hanley AB**, Burch R. Re: The role of STAT-3 in the induction of apoptosis in pancreatic cancer cells by benzyl isothiocyanate. *J Natl Cancer Inst* 2009; **101**: 893; author reply 893-894
65 **Schutte M**, Rozenblum E, Moskaluk CA, Guan X, Hoque AT, Hahn SA, da Costa LT, de Jong PJ, Kern SE. An integrated high-resolution physical map of the DPC/BRCA2 region at chromosome 13q12. *Cancer Res* 1995; **55**: 4570-4574
66 **Levy L**, Hill CS. Smad4 dependency defines two classes of transforming growth factor {beta} (TGF-{beta}) target genes and distinguishes TGF-{beta}-induced epithelial-mesenchymal transition from its antiproliferative and migratory responses. *Mol Cell Biol* 2005; **25**: 8108-8125
67 **Dessimoz J**, Grapin-Botton A. Pancreas development and cancer: Wnt/beta-catenin at issue... *Cell Cycle* 2006; **5**: 7-10
68 **Rulifson IC**, Karnik SK, Heiser PW, ten Berge D, Chen H,

Gu X, Taketo MM, Nusse R, Hebrok M, Kim SK. Wnt signaling regulates pancreatic beta cell proliferation. *Proc Natl Acad Sci USA* 2007; **104**: 6247-6252

69 **Al-Aynati MM**, Radulovich N, Riddell RH, Tsao MS. Epithelial-cadherin and beta-catenin expression changes in pancreatic intraepithelial neoplasia. *Clin Cancer Res* 2004; **10**: 1235-1240

70 **Rozenblum E**, Schutte M, Goggins M, Hahn SA, Panzer S, Zahurak M, Goodman SN, Sohn TA, Hruban RH, Yeo CJ, Kern SE. Tumor-suppressive pathways in pancreatic carcinoma. *Cancer Res* 1997; **57**: 1731-1734

71 **Sasaki S**, Yamamoto H, Kaneto H, Ozeki I, Adachi Y, Takagi H, Matsumoto T, Itoh H, Nagakawa T, Miyakawa H, Muraoka S, Fujinaga A, Suga T, Satoh M, Itoh F, Endo T, Imai K. Differential roles of alterations of p53, p16, and SMAD4 expression in the progression of intraductal papillary-mucinous tumors of the pancreas. *Oncol Rep* 2003; **10**: 21-25

72 **Poley JW**, Kluijt I, Gouma DJ, Harinck F, Wagner A, Aalfs C, van Eijck CH, Cats A, Kuipers EJ, Nio Y, Fockens P, Bruno MJ. The yield of first-time endoscopic ultrasonography in screening individuals at a high risk of developing pancreatic cancer. *Am J Gastroenterol* 2009; **104**: 2175-2181

73 **Harsha HC**, Kandasamy K, Ranganathan P, Rani S, Ramabadran S, Gollapudi S, Balakrishnan L, Dwivedi SB, Telikicherla D, Selvan LD, Goel R, Mathivanan S, Marimuthu A, Kashyap M, Vizza RF, Mayer RJ, Decaprio JA, Srivastava S, Hanash SM, Hruban RH, Pandey A. A compendium of potential biomarkers of pancreatic cancer. *PLoS Med* 2009; **6**: e1000046

74 **Vestergaard EM**, Hein HO, Meyer H, Grunnet N, Jørgensen J, Wolf H, Orntoft TF. Reference values and biological variation for tumor marker CA 19-9 in serum for different Lewis and secretor genotypes and evaluation of secretor and Lewis genotyping in a Caucasian population. *Clin Chem* 1999; **45**: 54-61

75 **Bussom S**, Saif MW. Methods and rationale for the early detection of pancreatic cancer. Highlights from the "2010 ASCO Gastrointestinal Cancers Symposium". Orlando, FL, USA. January 22-24, 2010. *JOP* 2010; **11**: 128-130

76 **Brand RE**, Lerch MM, Rubinstein WS, Neoptolemos JP, Whitcomb DC, Hruban RH, Brentnall TA, Lynch HT, Canto MI. Advances in counselling and surveillance of patients at risk for pancreatic cancer. *Gut* 2007; **56**: 1460-1469

77 **Sakorafas GH**, Sarr MG. Pancreatic cancer after surgery for chronic pancreatitis. *Dis Liver Dis* 2003; **35**: 482-485

78 **Wong T**, Howes N, Threadgold J, Smart HL, Lombard MG, Gilmore I, Sutton R, Greenhalf W, Ellis I, Neoptolemos JP. Molecular diagnosis of early pancreatic ductal adenocarcinoma in high-risk patients. *Pancreatology* 2001; **1**: 486-509

79 **Rosty C**, Geradts J, Sato N, Wilentz RE, Roberts H, Sohn T, Cameron JL, Yeo CJ, Hruban RH, Goggins M. p16 Inactivation in pancreatic intraepithelial neoplasias (PanINs) arising in patients with chronic pancreatitis. *Am J Surg Pathol* 2003; **27**: 1495-1501

80 **Ockenga J**, Vogel A, Teich N, Keim V, Manns MP, Strassburg CP. UDP glucuronosyltransferase (UGT1A7) gene polymorphisms increase the risk of chronic pancreatitis and pancreatic cancer. *Gastroenterology* 2003; **124**: 1802-1808

81 **Salek C**, Benesova L, Zavoral M, Nosek V, Kasperova L, Ryska M, Strnad R, Traboulsi E, Minarik M. Evaluation of clinical relevance of examining K-ras, p16 and p53 mutations along with allelic losses at 9p and 18q in EUS-guided fine needle aspiration samples of patients with chronic pancreatitis and pancreatic cancer. *World J Gastroenterol* 2007; **13**: 3714-3720

82 **Li D**, Yeung SC, Hassan MM, Konopleva M, Abbruzzese JL. Antidiabetic therapies affect risk of pancreatic cancer. *Gastroenterology* 2009; **137**: 482-488

S- Editor Tian L **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2909

World J Gastroenterol 2011 June 28; 17(24): 2909-2923
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2011 Baishideng. All rights reserved.

ORIGINAL ARTICLE

Genomic imbalances in esophageal carcinoma cell lines involve Wnt pathway genes

Jacqueline Brown, Hannelie Bothma, Robin Veale, Pascale Willem

Jacqueline Brown, Hannelie Bothma, Pascale Willem, National Health Laboratory Services and University of the Witwatersrand, York Rd, Parktown Johannesburg 2193, South Africa
Robin Veale, School of Molecular and Cell Biology, University of the Witwatersrand, Yale Rd, Johannesburg 2193, South Africa
Author contributions: Brown J performed most experiments, did the analysis and wrote this manuscript; Bothma H performed analysis of conventional cytogenetics; Veale R provided and cultured the cell lines; Willem P conceptualized the study, assisted with results analysis and wrote this manuscript.
Supported by The Cancer Association of South Africa, the National Health Laboratory Services Research Trust and the Medical Research council of South Africa
Correspondence to: Jacqueline Brown, MSc, National Health Laboratory Services and University of the Witwatersrand, York Rd, Parktown Johannesburg 2193,
South Africa. jacqueline.brown@nhls.ac.za
Telephone: +27-11-4898575 Fax: +27-11-4898480
Received: October 7, 2010 Revised: October 30, 2010
Accepted: November 6, 2010
Published online: June 28, 2011

Abstract

AIM: To identify molecular markers shared across South African esophageal squamous cell carcinoma (ESCC) cell lines using cytogenetics, fluorescence *in situ* hybridization (FISH) and single nucleotide polymorphism (SNP) array copy number analysis.

METHODS: We used conventional cytogenetics, FISH, and multicolor FISH to characterize the chromosomal rearrangements of five ESCC cell lines established in South Africa. The whole genome copy number profile was established from 250K SNP arrays, and data was analyzed with the CNAT 4.0 and GISTIC software.

RESULTS: We detected common translocation breakpoints involving chromosomes 1p11-12 and 3p11.2, the latter correlated with the deletion, or interruption of the *EPHA3* gene. The most significant amplifications involved the following chromosomal regions and genes: 11q13.3 (*CCND1*, *FGF3*, *FGF4*, *FGF19*, *MYEOV*), 8q24.21(*C-MYC*, *FAM84B*), 11q22.1-q22.3 (*BIRC2*, *BIRC3*), 5p15.2 (*CTNND2*), 3q11.2-q12.2 (*MINA*) and 18p11.32 (*TYMS*, *YES1*). The significant deletions included 1p31.2-p31.1 (*CTH*, *GADD45α*, *DIRAS3*), 2q22.1 (*LRP1B*), 3p12.1-p14.2 (*FHIT*), 4q22.1-q32.1 (*CASP6*, *SMAD1*), 8p23.2-q11.1 (*BNIP3L*) and 18q21.1-q21.2 (*SMAD4*, *DCC*). The 3p11.2 translocation breakpoint was shared across four cell lines, supporting a role for genes involved at this site, in particular, the *EPHA3* gene which has previously been reported to be deleted in ESCC.

CONCLUSION: The finding that a significant number of genes that were amplified (*FGF3*, *FGF4*, *FGF19*, *CCND1* and *C-MYC*) or deleted (*SFRP2* gene) are involved in the Wnt and fibroblast growth factor signaling pathways, suggests that these pathways may be activated in these cell lines.

© 2011 Baishideng. All rights reserved.

Key words: Esophagus; Cancer; Single nucleotide polymorphism arrays; Fluorescent *in situ* hybridization

Peer reviewer: Julian Swierczynski, MD, PhD, Professor, Department of Biochemistry, Medical University of Gdansk, 80-211 Gdansk, Poland

Brown J, Bothma H, Veale R, Willem P. Genomic imbalances in esophageal carcinoma cell lines involve Wnt pathway genes. *World J Gastroenterol* 2011; 17(24): 2909-2923 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2909.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2909

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is a major cause of cancer-related death in the world and it is char-

acterized by a peculiar epidemiology with worldwide geographic pockets of high incidence. In South Africa, the region of the Eastern Cape shows the highest incidence and ESCC represents the leading cancer affecting men and the second most common cancer in woman with a prevalence of 31.3 and 18 per 100 000 individuals, respectively[1]. The Gauteng (Johannesburg) and the Western Cape regions are also affected but to a lesser extent[2,3]. The tumors are diagnosed at advanced stages of the disease and patients have a poor survival rate. The etiology of this cancer is unresolved and while the most common risk factors associated with ESCC include smoking and alcohol consumption, these factors are surprisingly lacking in some parts of the world that have a high incidence such as in Iran and northern parts of China[4]. Additional risk factors have been proposed to play a role in some regions. In particular, exposure to fumonisin, a Fusarium fungi toxin that grows on maize, was reported in South Africa and China[5,6] as well as human papillomavirus (HPV) infection[7]. Poor nutrition is associated with ESCC in most parts of the world[4], and chronic inflammation was described in endemic parts of South Africa[8]. The respective part played by environmental risk factors and potential genetic susceptibility remains unclear and it is possible that different combinations of factors may be at play in different parts of the world.

It is widely accepted that recurrent chromosomal breakpoints in malignancies often pinpoint genes involved in the initiation or progression of cancer[9]. A major limitation in assessment of the chromosome complement in ESCC specimens is the difficulty in obtaining metaphases from fresh tumors, and established cell lines provide a unique resource for such investigations. The number of ESCC cell lines that have been reported to date remains limited and were all established in China[10-12] and Japan[13]. These cell lines have been investigated with one or several low resolution molecular cytogenetic techniques including cytogenetics, fluorescence *in situ* hybridization (FISH), multicolor FISH (M-FISH) or SKY and conventional comparative genomic hybridization (CGH). Various clonal aberrations have been identified and the most common abnormalities across studies involved over representation of chromosomes 1q, 3q, 11q and 8q as well as breakpoints in the centromeric or near centromeric regions of chromosomes 1, 3 and 8[10-12,14].

Five cell lines have previously been established from South African ESCC patients[15,16] but apart from cell line SNO, which was karyotyped, these cell lines were never characterized for their genetic constitution. We used conventional cytogenetics, FISH, and M-FISH to identify common chromosome structural abnormalities across these cell lines. Affymetrix 250K single nucleotide polymorphism (SNP) arrays were performed to investigate DNA copy number changes and the common aberrations detected by M-FISH and conventional cytogenetics. Here we describe clonal aberrations shared by these cell lines and highlight preferential targets for chromosomal rearrangements and copy number changes. These are the first ESCC cell lines from Africa to be genetically characterized to our knowledge.

MATERIALS AND METHODS

Cell lines

The five esophageal carcinoma cell lines used in this study were previously isolated from male patients with moderately differentiated ESCCs[16]. These cell lines were previously described and are designated as WHCO1, WHCO3, WHCO5, WHCO6 and SNO cell lines[15,16].

Cytogenetics

Cell lines were cultured at 37℃, 5% CO_2 in Dulbecco's Modified Eagles medium: HAMS F12 (3:1) (GIBCO®, Invitrogen Corporation, USA) containing 10% fetal calf serum, 100 μg/mL streptomycin (ICN, Costa Mesa, CA, USA) and 100 IU/mL of penicillin (ICN, Costa Mesa, CA, USA). When the cultures were half confluent, the cells were incubated with a final concentration of 0.44 μg/mL of Karyomax® Colcemid® (Invitrogen Corporation, USA) for 4 h. Cells were then harvested by standard cytogenetic procedure. Slides were either prepared for metaphase analysis using GTG banding by standard procedures or for FISH preparations.

M-FISH

M-FISH was performed using the SpectraVysion™ Assay (Vysis®, Abbott Molecular Inc, IL, USA) according to the manufacturer's protocol. The slides were analyzed on an Olympus BX41 fluorescent microscope with six single band pass filters for visualization of the six fluorophores. Genus™ CytoVision 3.0 software (Applied Imaging Corporation, San Jose, California, USA) was used for image acquisition and analysis. Ten metaphases were analyzed per cell line.

FISH

FISH was performed on metaphase chromosomes from all cell lines in order to confirm or refine translocation derivative breakpoints and clarify their composition. Probes specific for the short and long arms of chromosome 3, the short arm of chromosome 1 and the long arm of chromosome 22 (Qbiogene, Strasbourg, France) as well as the Vysis® Cep 3 Alpha and Cep 1 Alpha SpectrumOrange probes (Abbott Molecular Inc., IL, USA) were hybridized to further map the breakpoints. The Vysis® LSI IGH and Vysis® LSI RARA, both dual color break apart rearrangement probes (Abbott Molecular Inc., IL, USA) were used to confirm the involvement of chromosomes 14 and 15 in translocation derivatives seen in cell lines WHCO1 and WHCO3 respectively. In order to establish if the *EGFR* (epidermal growth factor receptor) gene was involved in a marker chromosome 7 the Vysis® LSI EGFR SO/CEP 7 SG probe (Abbott Molecular Inc., IL, USA) was hybridized to metaphase chromosomes.

FISH was also performed on interphase nuclei from all cell lines using the Vysis® LSI C-MYC SpectrumOrange

probe and the Vysis® LSI t(11;14) dual color probe (Abbott Molecular Inc., IL, USA). These probes target the *C-MYC* gene (Spectrum Orange) on 8q24 and the *CCND1* gene (Spectrum Orange) on chromosome 11q respectively and were used to confirm SNP array copy number results. A hundred interphase nuclei were analyzed in each cell line. All FISH experiments were performed according to the manufacturer's instructions and analyzed on an Olympus BX41 fluorescent microscope equipped with appropriate fluorescence filters. The Genus™ CytoVision 3.0 software was used for image acquisition and analysis.

DNA isolation

DNA was extracted from the cell lines and from six blood specimens obtained from healthy and population-matched volunteers using standard phenol-chloroform extraction methods. DNA was quantified on the ND-1000 Spectrophotometer (Nanodrop® Technologies, Rockland, DE) and quality assessed by gel electrophoresis.

SNP arrays and data analysis

Cell lines and control DNA were hybridized to the Affymetrix® GeneChip® Human Mapping 250 K Nsp Arrays (Santa Clara, CA). The GeneChip® mapping 500 K protocols were used. The control group used as a reference for analysis was DNA samples extracted from whole blood from black male volunteers.

Data analysis was performed using the Affymetrix®, Genotyping Console™ 2.0 and the copy number analysis tool (CNAT 4.0) software (Affymetrix®, Santa Clara, USA). Subsequently data were analyzed with third party software, Genepattern[17]. The signal intensities from the CEL files were normalized by the PM-MM (perfect match minus mismatch) probe intensity and quantile normalization against the median intensity of the controls. The raw copy number was then estimated as a ratio against the signal intensities of the normal reference samples. The log2ratios were smoothed using GLAD (Gain and Loss analysis of DNA)[18].

To determine the significant common regions of amplification and deletion (i.e. driver aberrations as opposed to random passenger aberrations) across the five cell lines, the GISTIC (Genomic Identification of Significant Targets in Cancer) algorithm[19] was applied to the smoothed data. The algorithm first scores the regions of copy number change according to their frequency and amplitude, which indicates the likelihood of these regions to represent a driver aberration (G-score). The statistical significance of each G-score is calculated by comparison of these scores against a null model of random aberrations. This significance is represented as a q-value (False discovery rate), which is the likelihood that the data was generated by chance. The most probable locations for oncogenes or tumor suppressor genes are identified by calculating the minimal common regions of aberration, which are most significantly altered i.e. high amplitude change. The regions of aberrations with high G-scores and minimal q-values (less than 0.25) are therefore more

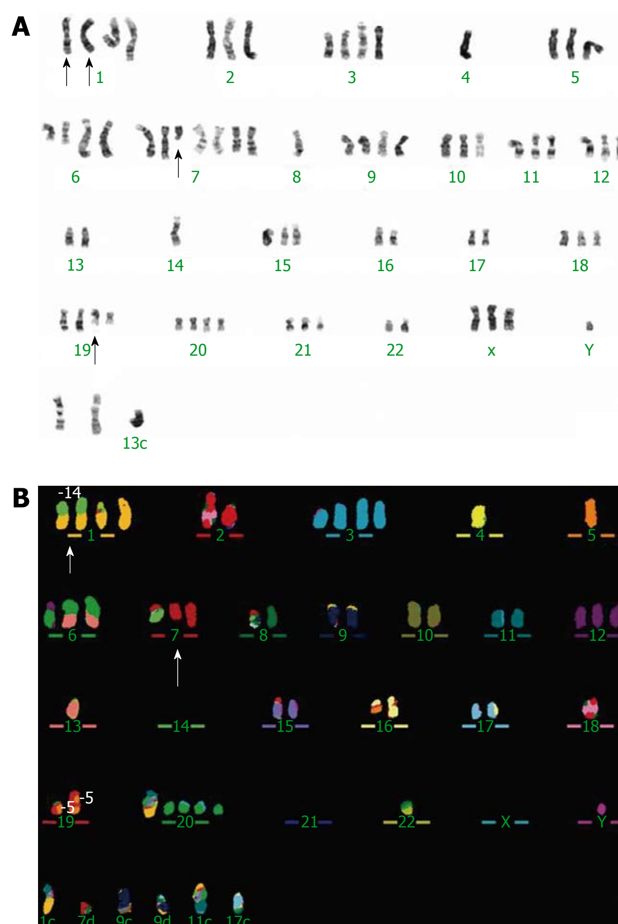


Figure 1 G-banded and multicolor fluorescence *in situ* hybridization representative karyotypes of cell line WHCO1. A: G-banded karyotype; B: Multicolor fluorescence *in situ* hybridization (M-FISH) karyotype, the arrows indicate the marker chromosomes, der(1) t(1;14)(p11;q11) and del(7)(q21).

likely to contain target genes that have a significant role in carcinogenesis. The chromosomal regions are then classified as high or low level where low level amplification is considered for a log2ratio greater than 0.1 but smaller than 0.9 and high level amplification is considered for a log2ratio > 0.9 (0.9 corresponds to at least 3.7 copies per diploid cell). Similarly low level deletions (hemizygous) were considered for a log2ratio of -0.1 and homozygous deletions were considered for a log2ratio < -1. The GISTIC algorithm has been used for copy number analysis in previous studies[20,21].

RESULTS

Cytogenetics and M-FISH

Cytogenetic analysis revealed complex numerical and structural chromosome aberrations in all cell lines with a high variability observed between cells from the same cell line (Figures 1 and 2). The G-banded karyotype of cell line WHCO1 and a corresponding M-FISH karyotype that illustrates the degree of variation from one metaphase to another are shown in Figure 1. The M-FISH data confirmed the complexity of the karyotypes and revealed

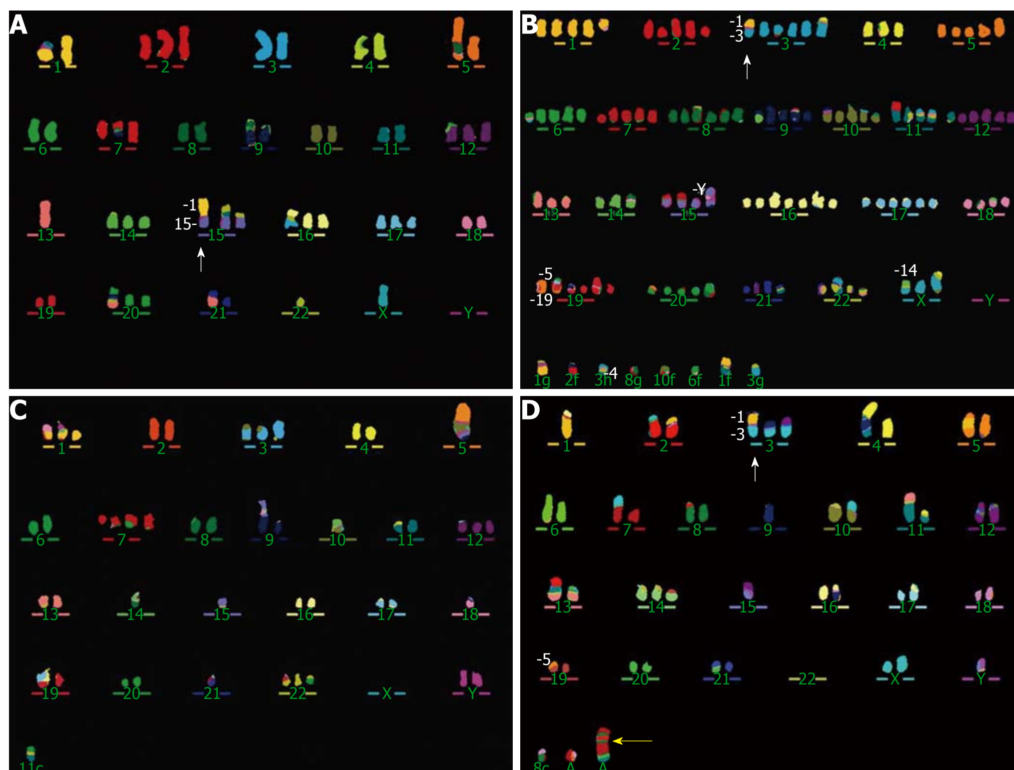


Figure 2 Multicolor fluorescence *in situ* hybridization representative karyotypes showing complex rearrangements in four cell lines. A: Cell line WHCO3, the white arrow indicates the der(15)t(1;15)(q11;p11); B: Cell line WHCO5, the white arrow points to the der(3)t(1;3)(p11-12;q11); C: Cell line WHCO6; D: Cell line SNO, the white arrow points to the der(3)t(1;3)(p11-12;p11) and the yellow arrow indicates the marker chromosome 7, mar(7), which involves the *EGFR* (epidermal growth factor receptor) locus (Figure 6).

the genetic composition of recurrent chromosome markers that could not be identified on G-banded metaphases (Figure 2); some of these markers are further discussed below. The detailed composite karyotypes obtained from twenty metaphases in each cell line are summarized in Table 1. All cell lines were near diploid except for cell line WHCO5 which was near tetraploid (Figure 2). Across the five cell lines a total of 97 translocations, 19 trisomies and 11 monosomies were detected. The breakpoints amounted to 203, with 78 of these clustering around the centromeric regions of chromosomes. The chromosomes involving the highest number of abnormalities were chromosomes 1, 3, 5, 7, 8, 9, 10, 11, 13, 14, 15, 18, 19, 20 and 22. G-banded metaphases also revealed the presence of isochromosomes involving the D group acrocentric chromosomes in all the cell lines. In particular isochromosome 13q, i(13)(p10) was common to cell lines WHCO3, WHCO5 and WHCO6, isochromosome 14q, i(14)(p10) was common to cell lines WHCO1, WHCO5 and WHCO6, and isochromosome 15q, i(15)(p10) was seen in cell lines WHCO3 and WHCO5.

The combined results of cytogenetics, FISH and M-FISH revealed a common translocation derivative, der (3)t(1;3)(p11;q11) that combined chromosome 3 and chromosome 1 short arms in cell lines WHCO5 and SNO (Figures 2-4). The SNP array copy number data did not bring further information on these breakpoints, possibly due to a lack of SNP probes in this region.

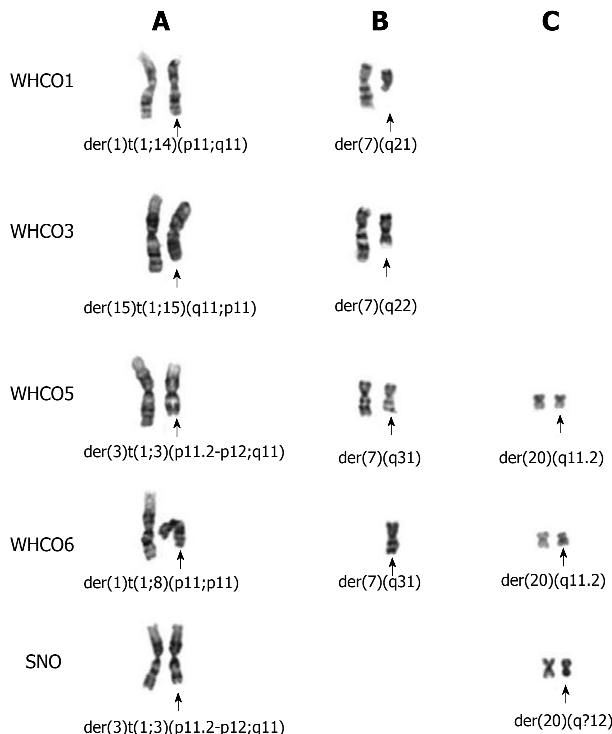


Figure 3 Partial G-banded karyotypes of the five cell lines showing shared chromosomal aberrations across cell lines. Chromosome derivatives from unbalanced translocations involving (A) chromosome 1p11-12 breakpoints in four cell lines, 1q11 in cell line WHCO3, (B) deletions of chromosome 7q in four cell lines, and (C) deletions of chromosome 20q in three cell lines.

Table 1 Composite karyotypes

Cell line	Composite combined cytogenetics and M-FISH karyotype
WHCO1	42~73, XY, +X, +X [4], +1 [10], del 1(?) [4], + der(1)t(1;14)(p11;q11) x2 [18], t(1;14;22)(?;?;q?) [4], t(1;22)(?;q?) [6], + 2 [8], t(2;8)(?;?) [4], +3 [8], +del 3(p21p13) x2 [4], der(3;22)(q10;q10) [4], -4 [8], t(5;21)(?;?) [8], + der(5)t(5;?)(q23;?) [4], + del(6)(q?21) x2 [4], t(6;12)(?q;?p) [16], t(6;13)(?q25;?q14) x2 [18], +7 [4], + del(7)(q21) [4], + der(7;14)(p10;q10) [20], der(7)t(7;18)(p21;q23) [16], der(7)t(7;19)(p22;p13) [4], der(8)t(8;?19)(q?24;q?11) [4], t(8;22)(?;?) [18], +9 [10], + der(10)t(9;10)(q13;q?11.2) [4], +11 [8], t(11;17;20)(?;?;?) [4], +12 [10], t(13;22)(?;?) [4], add(14)(q?) [4], i(14)(q10) [6], der(15;19)(q10;q10) [20], der(16)t(5;16)(?;?) [8], +18 [4], der(19)t(5;19)(p?12;q11) [12], der(19) t(5,19)(q33;q13.4) [18], + der(19)t(9;19)(q?13;q?13) x2 [4], t(19;21)(?;?) [14], +20, +20 [20], + der (20)t(1;11;20)(?;?;?) [4], +1~7mar [20] [cp20]
WHCO3	46~50, X, -Y, +2 [12], der(5)t(5;8,18)(q?;?;?) [20], + del 7(q22) [4], der(7)t(7;9)(?;?) [6], t(7;9;16;18)(?;?;?;?) [14], t(7;15)(?;?) [4], +12 [6], der(12)t(6;12)(?;?) [16], i(13)(q10) [6], t(13;14)(?;?) [6], t(13;14;20)(?;?;?) [6], +14 [12], + der(15)t(1;15)(q11;p11) [6], + der(15)t(1;15;11)(?;?;?) [10], der(15;22)(q10;q10) [20], i(15)(q10) [4], der(16)t(3;16;22)(p?11.2;?;q?10) [20], +17 [10], der(?20)t(9;13;20)(?;?;?) [10], der(21)t(13;21)(?;?) [12] [cp20]
WHCO5[1]	99~108, XY, t(X;4;10.22)(?;?;?;q?) [14], t(1;19)(?;?) [8], t(1;18)(?;?) [6], del 2 [2], der(2)t(2;9)(q12;q13) [10], t(2;9)(?q31;?q34) [8], +der(3)t(1;3)(p11-12;q11) [12], t(3;11;13;22)(?;?;?;?) [8], t(3;11;22)(?;?;?) [16], t(3;22)(p11;q11) [12], der(5;20)(p10;p10) [4], t(6;13)(?;?) [4], del(7)(q31) [4]; t(8;14;18)(?;?;?) [16], t(8;18)(?;?) [10], der(9)t(9;14)(?;?) [16], t(9;15)(q?;q?) [10], t(9;19)(?;?) [6], t(12;19)(?;?) [4], i(13)(q10) [6], i(14)(q10) [8], der(15)t(Y;15)(?;?)[12], der(15)t(7;15)(?;?) x2 [14], i(15)(q10) [4], der(19)del(19)(q13.?2)t(5;19)(p?12;p11) [12], del(20)(q11.2) [12] [cp20]
WHCO6	44~54, Y, -X, der(1)t(1;8)(p11;q11) [14], t(3;10)(?;?) [4], t(3;10)(?q13.3;p10) [6], t(4;10)(?;?) [4], t(5;10)(?;?) [10], t(5;22)(?;q?) [4], +6 [2], del(6)(q?21) [4]; t(6;11)(p12;q13) [6], der(22)t(6;22)(?;?) [6], +7[7], del(7)(q31) [8]; +8 [8], t(9;15)(?;?) [10], t(10;14)(?;?) [6], -11 [6], t(11;22)(p?;q?) [6], +12 [10], i(13)(q10) [6]; i(14)(q10) [6], +16 [8], t(17;19)(?;?) [10], +18 [4], del(20)(q?11.2) [6]; -21 [12], t(21;22)(?;?) [6], der(22)t(6;22)(?;?) [6], [cp14]
SNO	29~43, XY, +X, del X(?) [14], der(Y;15)(q10;q10) [12], t(1;16)(?;?) [14], der(2)t(X;2)(?;?) [16], der(2)t(1;2)(?;?) [18], der(3)t(1;3)(p11-12;q11) [20], t(3;9)(?;?) [4], t(3;10)(?;?) [14], t(3;12)(?;?) [20], t(3;20)(?;?) [8], t(4;9;11)(?;?;?) [20], t(4;11)(?;?) [12], der(5)t(1;5)(?;?) [20], -6 [18], del(6)(q?23) [20], der(7)t(3;7)(?q25;p22) [18], t(7;20;11;8;2)(?;?;?;?;?) [18], der(8)t(2;8)(?;?) [14], t(8;18)(?;?) [8], -9 [10], t(9;18)(?;?) [4], t(10;22)(q?;q?) [12], t(11;13)(?;?)[8], t(12;15)(?;?)[8], t(12;21)(?;?)[20], t(13;11;20)(?;?;?) [14], der(14;22)(p10;q10) x2 [20], t(14;19)(?;?) [20], -16 [14], der(16) t(9;16)(q?22;q?13) [20], der(17)t(6;17)(?;?) [12], -18 [16], -19 [16], der(19)del(19)(q13.?2)t(5;19)(q?12;q10) [16], +20 [10], del(20)(q?12) [16]; der(20;21)(q10;p10) [6], -21 [18], -22 [20] [cp20]

[1]Only structural rearrangements are listed due to the complexity of this cell line.

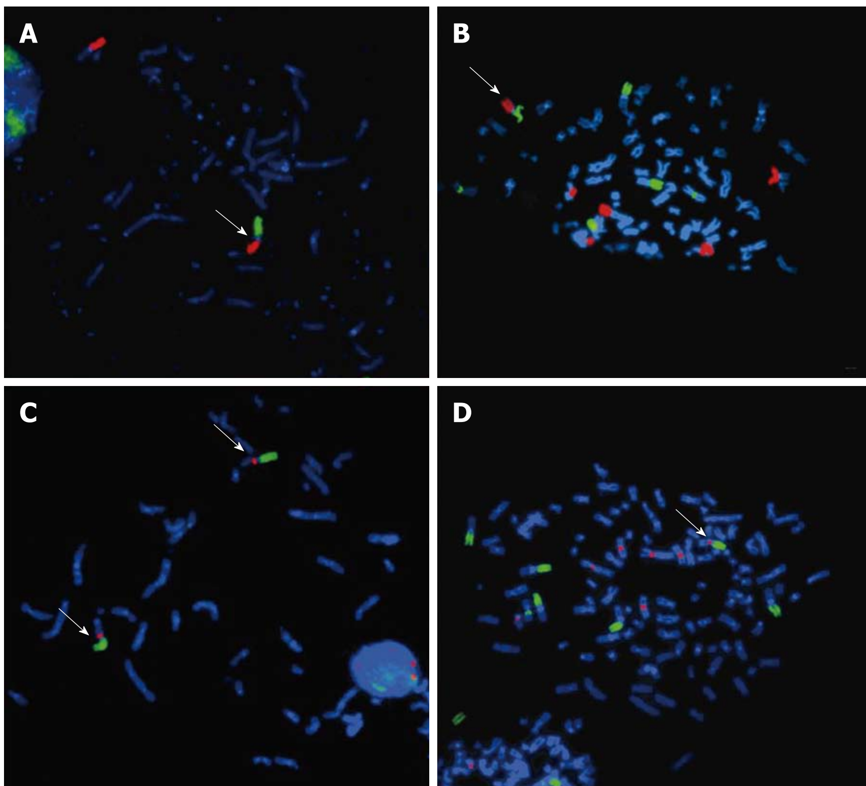


Figure 4 DAPI stained metaphasic chromosomes showing the der(3)t(1;3)(p11-12;q11) in cell lines SNO and WHCO5. A, B: Arm-specific paint for chromosomes 1p (green) and 3p (red) in cell lines SNO and WHCO5 respectively showing the derivatives der(3)t(1;3)(p11-12;q11) arrowed; C, D: Arm specific paint for chromosome 1p (green) and Cep 3 α probe (red) in cell lines SNO and WHCO5 respectively showing that the centromere of chromosome 3 is retained on the derivatives der(3)t(1;3)(p11-12;q11).

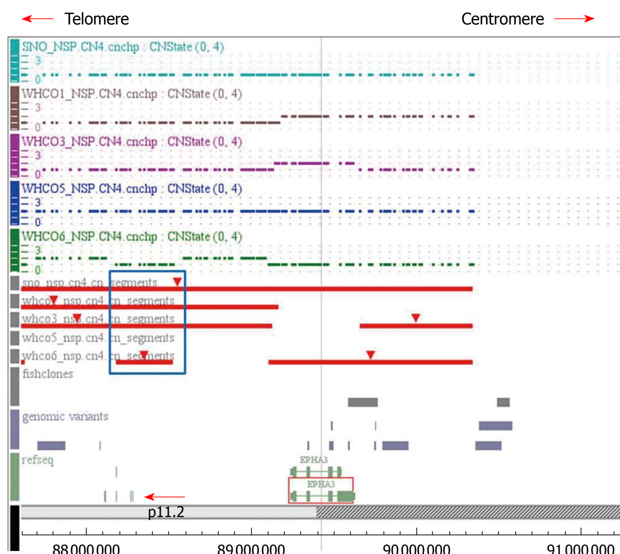


Figure 5 CNAT plot of single nucleotide polymorphism hybridization results showing the map of the region of deletion on chromosome 3p11.2-12.1 in cell lines WHCO1, WHCO3, WHCO5, WHCO6 and SNO. The red bars indicate the segment of deletion as determined by CNAT 4.0. The dotted lines indicate the copy number status for each single nucleotide polymorphism probe for each cell line. The blue block indicates the minimal common region of deletion and the red arrow indicates the two genes in this region, *c3orf38* and *CGGBP1*.

Interestingly, the 1p11 breakpoint was also involved in cell lines WHCO1 and WHCO6 in differing unbalanced translocations (Figure 3). The M-FISH and FISH results with probes for the respective partner chromosomes and centromeric Cep1, confirmed the interpretation of these derivatives as der(1)t(1;14)(p11;q11) in cell line WHCO1 and der(1)t(1;8)(p11;p11) in cell line WHCO6 (results not shown). In contrast, a translocation derivative also involving the chromosome 1 pericentromeric region in cell line WHCO3 (Figure 3) was shown not to involve 1p11 but 1q11, and was interpreted as der(15)t(1;15)(q11-12;p11) (result not shown).

Translocation derivatives involving chromosomes 3 and 22 were seen in cell lines WHCO3 and WHCO5 and were interpreted as der(16)t(3;16;22)(p11;?;q?11) and t(3;22)(p11;q11) respectively (Table 1). The array data showed corresponding hemizygous deletions at 3p11.2, in cell line WHCO3. Interestingly deletions at 3p11.2 were also observed in cell lines WHCO1, SNO and WHCO6. The minimal region of overlap was 343 kb (88184220-88527215 bp) in size and involved *c3orf38* and CGG triplet repeat binding protein (*CGGBP1)* genes. In addition the *EPHA3* gene was deleted in cell lines WHCO6 and SNO (Figure 5). The *EPHA3* gene encodes a tyrosine kinase, which is mutated in lung and breast cancers[22-24].

Cell lines WHCO1, WHCO3, WHCO5 and WHCO6 all showed a deletion of chromosome 7 long arm with varying breakpoints, q21 to q31, on G-banded metaphases (Figure 3). However GISTIC analysis of the SNP array data identified a significant common focal deletion (q-value of 0.09) of approximately 5.16 Mb at 7q33-q34 (133721542-138880555 bp) in only three of these cell lines. This region contained 26 genes including the potential target gene homeodomain-interacting protein kinase-2 (*HIPK2)* the product of which activates p53 expression and is pro apoptotic[25].

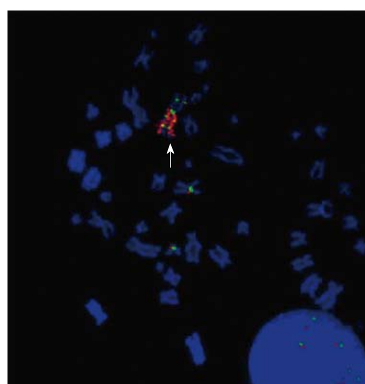


Figure 6 DAPI stained metaphase from cell line SNO showing fluorescence *in situ* hybridization results with the locus specific EGFR probe (red) and Cep7 α (green). The arrow indicates the chromosome 7 marker with a homogeneously stained region that contains the *EGFR* locus.

Although deletions at 20q11.2 were detected in cell lines WHCO5, WHCO6 and at 20q12 in cell line SNO on G-banded metaphases (Figure 3), copy number analysis (CNAT) revealed that there was in fact amplification of 20q sequences in all cell lines implying that complex rearrangement occurred for these sequences to be relocated elsewhere in the genome. Two minimal regions of amplification were identified, the 20q11.21-q11.22, of approximately 1.12 Mb in size (31799867-32906584 bp), which contained 14 genes and a smaller region at 20q13.12 of 149.48 kb which contained five genes. Both these regions were amplified in all cell lines, yet these amplifications were not found to be significant on GISTIC analysis.

Cell line SNO showed a large marker chromosome 7 on metaphases analyzed by M-FISH. This marker was interpreted as a possible inverted duplication of chromosome 7p sequences (Figure 2D). FISH with an *EGFR* probe, revealed a high amplification of the *EGFR* gene in 14% of the cells (Figure 6). A high level amplification at 7p13-7p11.2 (genomic location of *EGFR*) was confirmed by GISTIC (q-value 0.14) on array analysis in this cell line, while low level amplification was observed in the remaining four cell lines in agreement with the presence of 4 to 7 copies on FISH analysis.

Copy number changes

In view of the high cell to cell heterogeneity in each cell line, and in order to separate the potential driver aberrations out of the background of aberrations that may have occurred by chance, we used the software GISTIC specifically designed for this purpose. Target genes, defined as genes whose alteration confers a cell growth advantage, are likely to reside in the regions amplified or deleted to the highest degree in a majority of cells and within a common region of overlap amongst all cell lines. Fourteen common regions of amplification and 20 regions of deletion were

Table 2 Amplification peaks as detected by the GISTIC algorithm

Cytoband	Location (kb)	Approx size (Mb)	Frequency (*n*/5)	Mean log2ratio[1]	q-value	Genes[2]
1p34.2	Chr1:39027237-40780163	1.75	3	1.10	0.18	*MYCL1*
3q11.2-q12.2	Chr3:95917505-101945216	6.02	4	1.40	0.17	*MINA*
5p15.2	Chr5:10051329-11800765	1.75	2	1.36	0.09	*CTNND2*
7p11.2-p13	Chr7:45294289-57299457	12.01	1	1.24	0.13	*EGFR*
8q24.21	Chr8:127445828-129661846	2.22	5	2.10	0.0007	*MYC*
9q31.1	Chr9:100905143-102152148	1.25	1	2.57	0.15	
10q12.33-q21.3	Chr10:17811791-65388337	47.58	1	3.05	0.13	
11q13.3	Chr11:68753086-69985447	1.23	4	1.81	$5.76E^{-05}$	*CCND1, CTTN, FGF3, FGF4, FGF19, MYEOV*
11q22.1-q22.3	Chr11:100815801-103042620	2.23	2	2.35	0.03	*BIRC2, BIRC3, YAP1*
18p11.32	Chr18:1-1118244	1.12	2	1.04	0.13	*TYMS, YES1*
20p11.1-p11.22	Chr20:22140447-26145930	4.01	2	1.24	0.13	*PYGB*
22q11.21	Chr22:16558724-17937900	1.38	2	1.20	0.21	*BID, CLDN5*
22q11.21-q11.22	Chr22:18577713-20667607	2.10	2	1.20	0.13	*CRKL, MAPK1*
22q12.3	Chr22:31889314-32003182	0.11	2	1.15	0.13	*LARGE*

[1]The mean log2ratio for the samples with a log2ratio > 0.9 (equivalent to 3.7 copies per diploid cell); [2]The selected genes from the peak region.

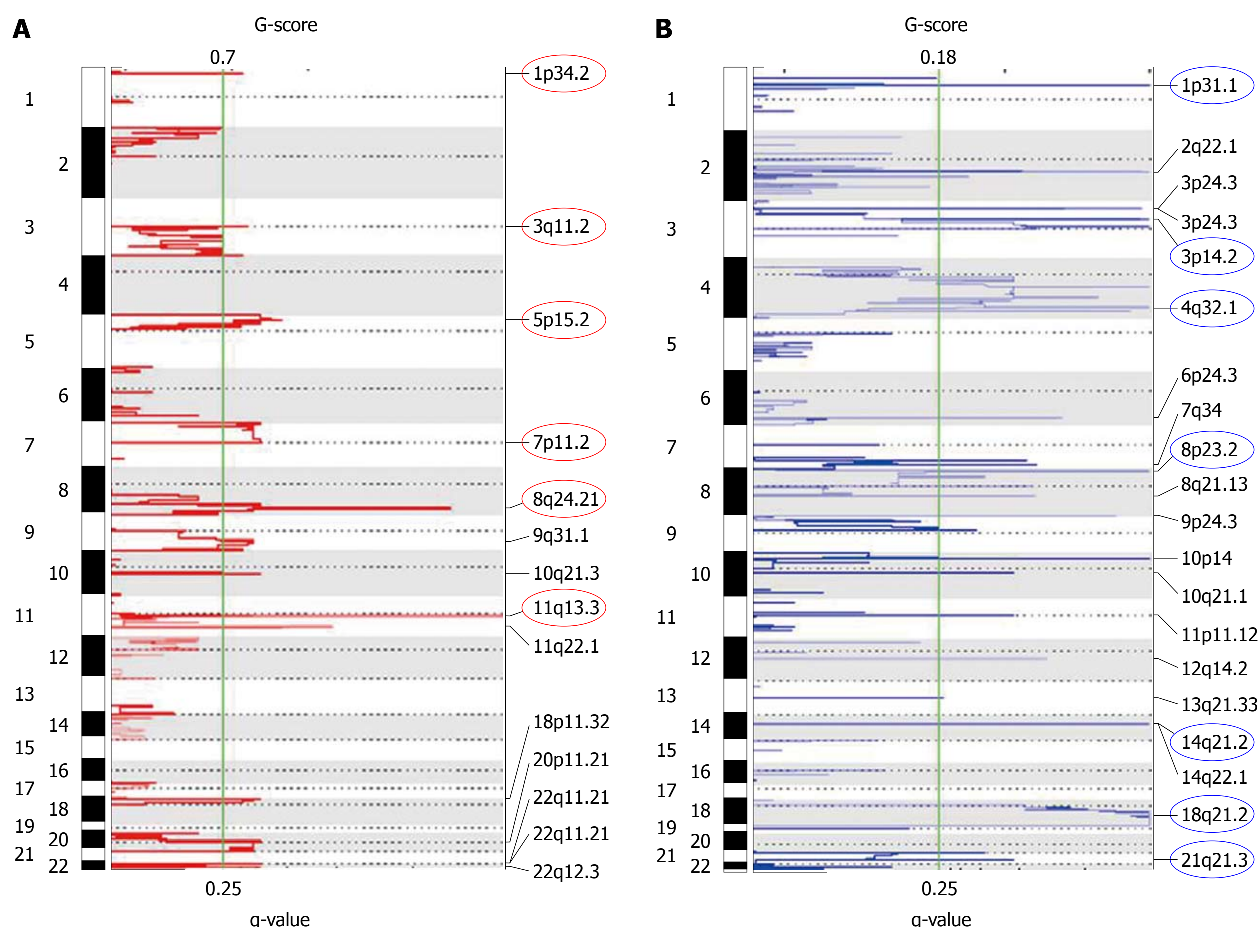


Figure 7 Plots of recurrent genomic amplifications (A) and deletions (B) detected in the esophageal squamous cell carcinoma cell lines from GISTIC analysis of single nucleotide polymorphism array data. The x-axis shows the G-score (top) and false discovery rate (q value; bottom). The green line indicates the false discovery rate cut off of 0.25. The circles indicate the peaks of the most significantly aberrant chromosomal regions.

identified (Figure 7, Tables 2 and 3). The most significant chromosomal regions of amplification were, in descending order of significance: 11q13.3 and 8q24.21, in four and five cell lines respectively, 11q22.1-q22.3 and 5p15.2 both detected in two cell lines. Chromosomal regions of less significant amplification were 10p12.33-q21.3, 18p11.32, 20p11.1-p11.22, 22q11.21-q11.22, 22q12.3, 9q31.1, 22q11.21, 3q11.2-q12.2 and 1p34.2 (Figure 7). Similarly, chromosomal regions of deletion were seen in the following order of significance: 1p31.1-p31.2, 2q22.1,

Table 3 Deletion peaks as detected by the GISTIC algorithm

Cytoband	Location (kb)	Size (Mb)	Frequency (*n*/5)		Mean log2ratio[1]	q-value	Genes[2]
			Hemi	Homo			
1p31.1-p31.2	Chr1:66691991-71187083	4.50	3	1	-3.03	0.02	*CTH*
2q22.1	Chr2:141590067-141951947	0.36	3	2	-1.66	0.19	*LRP1B*
3p26.3-q29	Chr3:1-199344050	199.34	2	1	-2.90	0.022	
3p12.1-p14.2	Chr7:60424050-85108679	24.70	4	1	-2.99	0.02	*FHIT, ADAMTS9*
4q22.1-q32.1	Chr4:91972774-162358674	70.40	3	2	-2.19	0.02	*CASP6, SMAD1*
6q24.3-q27	Chr6:147967444-170914576	22.95	2	1	-2.22	0.07	
7q33-q34	Chr7:133721542-138880555	5.16	2	1	-1.72	0.09	*HIPK2*
8p23.2-q11.1	Chr8:4078057-47043375	43.00	4	1	-2.47	0.02	*BNIP3L, INDO*
8p23.3-q21.13	Chr8:1-146308819	146.31	1	1	-2.27	0.09	
9p24.2-p24.3	Chr9:1151516-2459741	1.31	2	1	-2.53	0.03	
10p12.31-p14	Chr10:10309026-19155158	408.85	2	1	-2.74	0.02	
10q11.23-q22.1	Chr10:50393324-70694787	20.30	1	1	-2.35	0.12	
11p11.12-q12.2	Chr11:50256798-61426521	11.20	1	1	-1.90	0.12	
12p13.33-q24.33	Chr12:1-132078379	132.10		1	-2.80	0.08	
13q21.33-q34	Chr13:68772537-113042980	44.30	1	1	-1.70	0.24	
14q21.2	Chr14:42828345-44176016	1.35	3	1	-4.50	0.02	
14p13-q32.33	Chr14:1-105311216	105.30		1	-5.10	0.02	
18q21.1-q21.2	Chr18:46081464-51919972	5.80	5		-1.03	0.02	*DCC, SMAD4*
21p13-q21.3	Chr21:1-29932926	29.90	3			0.12	*BAGE*

[1]The mean of the log2ratio of those samples with log2ratios < -1.3 (< 0.9 copies per diploid cell); [2]The selected genes within the deletion peaks.

Table 4 Fluorescence *in situ* hybridization for detection of *CCND1* and *C-MYC* amplification

Cell line	FISH signals	Amplitude[1]	Log2Ratio
CCND1			
WHCO1	5-15	2	1.56
WHCO3	> 20	2	2.57
WHCO5	10-20	1	0.78
WHCO6	4-8	1	0.55
SNO	15-20	1	0.79
C-MYC			
WHCO1	4-6	1	0.12
WHCO3	15-> 20	2	1.83
WHCO5	10-> 20	2	1.83
WHCO6	10-> 20	2	2.06
SNO	4-> 20	2	1.71

[1]Amplitude threshold where log2ratio < 0.1 = 0, log2ratio > 0.1 < 0.9 = 1 and log2ratio > 0.9 = 2 as determined by SNP array copy number analysis. FISH: Fluorescence *in situ* hybridization.

3p12.1-p14.2, 4q22.1-q32.1, 8q11.1-p23.2, 14q21.2, 18q21.1-q21.2 and 21p13-q21.3 detected in all or four cell lines. Other regions of less significant deletion, and only seen in one or two cell lines, included: 10p12.31-p14, 14p13-q32.33, 3p26.3-q29, 9p24.2-p24.3, 6q24.3-q27, 12p13.33-q24.33, 7q33-q34, 8p23.3-q24.3, 10q11.23-q22.1, 11p11.12-q12.2 and 13q21.33-q34 (Figure 7). Together the regions of amplification and deletion encompassed a total of 4595 genes.

Significant gains

We selectively describe below the five regions of amplification that were the most significant on GISTIC analysis (q-value < 0.25) (Figure 7). Chromosomes 11q13.3 (68 753 086-69 985 447 bp) and 8q24.21 (127 445 828-129 661 846 bp) were the two most amplified and most significant regions with a q-value of 5.76E^{-05} and 0.0007 respectively. The 11q13.3 region was 1.23 Mb in size and was highly amplified in cell lines WHCO3, WHCO5, WHCO6 and SNO while amplified to a lesser degree in cell line WHCO1. This region harbors seven candidate genes including the cyclin D1 (*CCND1*), the cortactin (*CTTN*), the protein tyrosine phosphatase, receptor type, polypeptide, interacting protein α 1 (*PPFLA1*), *FGF3*, *FGF4* and *FGF19* and the myeloma overexpressed (*MYEOV*) genes, which could all play a role in ESCC oncogenesis. FISH validated these findings and confirmed the amplification of *CCND1* (Table 4, Figure 8). The common amplicon of 2.22 Mb at 8q24.21 was highly amplified in cell lines WHCO1 and WHCO3 and moderately amplified in cell lines WHCO5, WHCO6 and SNO. This amplicon involved the oncogene v-myc myelocytomatosis viral oncogene homolog (*C-MYC*) and the family with sequence similarity 84, member B (*FAM84B*) gene. Locus specific FISH confirmed the amplification of *C-MYC* in cell lines WHCO1, WHCO3, WHCO5, WHCO6 and SNO (Table 4, Figure 8).

A second focal region of high amplification on chromosome 11 was observed at 11q22.1-q22.3 (2.23 Mb in size) in cell lines WHCO5 and SNO. This region included the regulators of apoptosis *BIRC2* (*cIAP1*) and *BIRC3* (*cIAP2*), the matrix metalloproteinases (*MMP*) and the Yes associated protein (*YAP-1*) genes all potential target genes. The *BIRC2* gene was previously described as a target of amplification /increased expression in cervical cancers[26] and the *YAP-1* gene product is a cellular adaptor protein, which can induce *BIRC2* expression. *YAP-1* was reported to be over expressed in hepatic and mammary cancers[27]. In turn, the MMP genes, which include

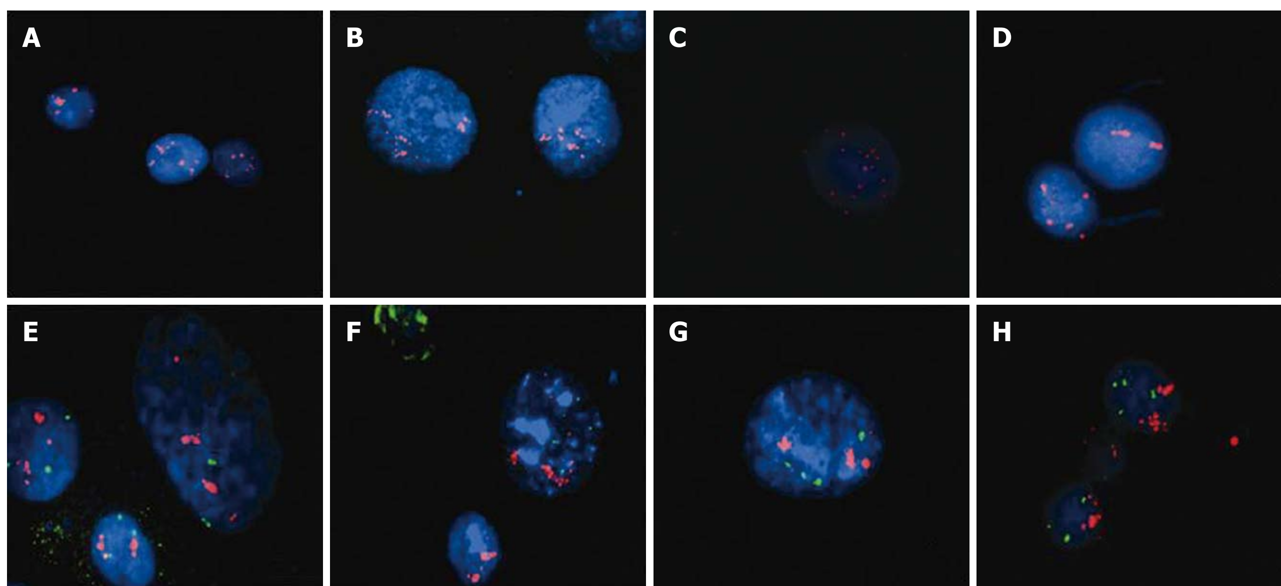


Figure 8 DAPI stained interphase nuclei hybridized with locus specific probes for C-MYC Spectrum Orange (red) (A-D) and *CCND1* (red)/*IGH* (green) (E-H). C-*MYC* amplification can be seen in cell lines WHCO1 (A), WHCO3 (B), WHCO5 (C) and SNO (D). *CCND1* amplification was detected in cell lines WHCO3 (E), WHCO5 (F), WHCO6 (G) and SNO (H).

MMP1, *MMP7*, and *MMP13*, have been shown to be co-expressed in early stage ESCC correlating with a poorer prognosis[28].

A 1.75 Mb region on chromosome 5p15.2 (10 051 329-11 800 765 bp) was highly amplified in cell lines WHCO6 and WHCO5 and moderately amplified in cell lines WHCO1 and SNO. This region hosts the potential target gene, delta catenin (*CTNND2*), overexpressed in prostate cancer[29].

Four cell lines, WHCO6, WHCO3, WHCO5 and SNO, had focal gain on chromosome 3q. The minimal common region of amplification mapped at 3q11.2-12.2, and was 6.02 Mb in size (95 917 505-101 945 216 bp) (q-value = 0.17). This region is commonly amplified in a variety of cancers[20,30] including esophageal squamous carcinoma[31] and it involved the potential oncogene, MYC induced nuclear antigen (*MINA*).

The 18p11.32 sub-band was highly amplified in cell lines WHCO3 and WHCO6 and moderately amplified in the remaining 3 cell lines. This region of 1.12 Mb in size (1-1 118 244 bp) has previously been described in ESCC[32] and involves the potential oncogenes *TYMS* and *YES-1*. Both genes have been implicated in gastro intestinal cancer. The *TYMS* gene codes for a thymidylate synthase involved in DNA synthesis and targeted by the chemotherapy agent fluorouracil (5FU). *TYMS* overexpression leads to 5FU treatment resistance[32] and affects colorectal cancer treatment[33]. *YES-1* is a homologue of the Yamaguchi sarcoma virus v-*yes* amplified and overexpressed in gastric cancers and ESCC[32].

Significant losses

The most significantly deleted chromosomal regions were 1p31.1-p31.2, 2q22.1, 3p12.1-p14.2, 4q22.1-q32.1, 8p23.2-q11.1, 14q21.2 and 18q21.1-q21.2 (in descending order of significance) in all or four of the cell lines (Figure 7). Less significant regions detected in less than four cell lines are depicted in Table 3. Three small regions of deletions involved chromosomes 1p, 2q and 18q. The 4.5 Mb deletion on chromosome 1 short arm, 1p31.2-p31.1 (66 691 991-71 187 083 bp) was seen in four cell lines (homozygous deletion in cell line SNO and hemizygous in cell lines WHCO1, WHCO5, WHCO6) with a high significant q-value of 0.02. Three genes with a reported tumor suppressor activity were involved in this deletion, the cystathionine γ-lyase (*CTH*), the growth arrest and DNA damage-45 α (*GADD45*α) and the DIRAS family, GTP-binding RAS-like 3 (*DIRAS3*) genes. The *DIRAS3* gene was shown to be down regulated in hepatocellular carcinoma and breast cancers[34,35] and is postulated to have a tumor suppressive activity. Both the *CTH* and *GADD45* genes were shown to negatively control cell growth[36,37].

Chromosome 2, sub-band q22.1 (141 590 067-141 951 947 bp) was lost in all cell lines (homozygous deletion in cell lines WHCO3 and WHCO5 and hemizygous in the other 3 cell lines) (q-value = 0.019). The low density lipoprotein 1B (*LRP1B*) tumor suppressor gene, deleted in lung cancer[38,39] maps in this region.

A chromosome 18q sub-band, q21.1-q21.2 (46 081 464-51 919 972 bp) (5.8 Mb) was hemizygously deleted in all five cell lines, involving both the *SMAD4* and deleted in colorectal carcinoma (*DCC*) genes.

Three large regions of deletion involved chromosomes 3p, 4q and 8p. First, the 24.7 Mb region of deletion at 3p12.1-p14.2 (60 424 050-85 108 679 bp) was significant (q-value of 0.02) in all cell lines (homozygous deletion in cell line SNO). This region houses the FRA3B associated gene, Fragile Histidine triad (*FHIT*), whose deletions were previously detected by MLPA analysis in these cell lines[40]. The potential tumor suppressor *ADAMTS9* gene, a me-

talloproteinase family member involved in inhibition of angiogenesis[41] was also involved in this deletion.

Second, the 4q22.1-q32.1 (91972774-162358674 bp) (75 Mb) region was homozygously deleted in cell lines WHCO3 and WHCO5 and hemizygously deleted in the three other cell lines with a q-value of 0.02. This region encompasses many genes but of interest are the Bone morphogenetic protein receptor 1B (*BMPR1B*), the caspase 6 (*CASP6*), the secreted frizzled-related protein 2 (*SFRP2*) and the SMAD protein 1 (*SMAD1*) genes, all potential tumor suppressor genes.

Lastly, a chromosome 8p23.2-q11.1 (4078057-47043375 bp) (43 Mb) deletion was seen in all cell lines (homozygous in cell line WHCO3 and hemizygous in the other 4 cell lines) (q value of 0.02), including five potential target genes, the BCL2/adenovirus E1B 19kDa interacting protein 3-like (*BNIP3L*), the leucine zipper tumor suppressor 1 (*LZTS1*) and the three tumor necrosis factor related superfamily genes *TNFRSF10A, TNFRSF10B* and *TNFRSF10C*.

DISCUSSION

We have characterized the karyotype and genomic constitution of five ESCC cell lines established in SA using a combination of traditional cytogenetics, M-FISH, FISH and SNP arrays. The number of ESCC cell lines genetically described worldwide is limited. Only eight ESCC cell lines have been investigated previously with traditional cytogenetics to our knowledge[10,12,42] and 10 with M-FISH[11,43,44], these are the only two techniques that can detect recurrent translocation breakpoints. In this study the five cell lines had complex karyotypes and were hyperploid with WHCO5 being near tetraploid. There was a high level of intra cell-line heterogeneity. The chromosomes most frequently involved in translocations were chromosomes 1, 3, 5, 7, 8, 9, 10, 11, 13, 14, 15, 18, 19, 20 and 22. These features were comparable to ESCC cell lines previously characterized[10-12,42,43] and across all studies that involved karyotyping, including the karyotyping of fresh ESCC tumor samples[14], chromosomes 1, 3 and 8 were the most commonly affected by translocation breakpoints[10-12,43].

Forty percent of translocation breakpoints occurred in near centromeric regions (Table 1). These were represented by unbalanced whole arm chromosome translocations, frequently involving chromosomes 1 and 3, and by isochromosomes for the D group acrocentric chromosomes 13, 14 and 15. Indeed, frequent centromeric breakpoints have been described in squamous carcinoma including ESCC[10,12] with up to 60% of all breakpoints being in centromeric regions[43] supporting the idea that centromeric disruption is a frequent event in epithelial cancers. It has been suggested that environmental factors may preferentially interact with centromeric sequences[45], and clastogenic compounds, such as mitomycin C, induce breaks in centromeric of chromosomes 1, 9 and 16[46]. Smoking is a major risk factor associated with ESCC and nicotine is known to induce single strand DNA breaks[47]. Although active smokers exhibit an increased number of breaks at fragile sites[48], it is not known if centromeric regions are also targeted.

Two chromosomal breakpoints were shared across cell lines. First, chromosome 1p11 was involved in a translocation in four cell lines, translocation t(1;3)(p11.2-12; q11) in cell lines SNO and WHCO5 and translocations with differing partners in cell lines WHCO1 and WHCO6. Second, chromosome 3p11-12 was involved in translocations in two cell lines and deletions in, or near the *EPHA3* locus was seen in four cell lines.

This gene codes for a receptor tyrosine kinase, with a tumor suppressor activity[49]. It was found to be mutated in lung and breast cancers[22-24] and interestingly, found to be deleted in 18.2% of ESCC patients in a previous study[31].

Breakpoints in, or near the centromeric regions of chromosomes 1 and 3 have previously been reported in several ESCC cell lines[10-12], as well as in fresh ESCC tumor samples that were karyotyped[14], and in ESCC cell lines obtained by *in vitro* transformation with HPV[50]. This strongly points to 1p11 and 3p11 translocation hotspots in ESCC that may affect genes and/or regulatory sequences not identified by SNP array. Deletions affecting or near the *EPHA3* gene may point to a role for this gene which was previously found to be deleted in 18.2% of ESCC patients[31].

We knew from previous studies that these ESCC cell lines, all overexpress the *EGFR* gene[16]. *EGFR* DNA amplification observed in cell line SNO is likely to contribute to *EGFR* overexpression whilst other factors are likely to be involved in the other four cell lines where low levels of *EGFR* amplification were observed.

In view of the high clonal heterogeneity observed in each cell line, we used the GISTIC software in addition to CNAT to analyze SNP array data and evidence the significant targets of amplification and deletions. The most stable genetic rearrangements are thought to reflect a proliferative cell growth advantage. In this context, the GISTIC algorithm allowed us to prioritize amplicons and regions of deletions in term of their likelihood to host driver genes. The five most interesting significant regions of amplification included chromosomal regions: 11q13.3, 8q24.21, 11q22.1-q22.3, 5p15.2 and 3q11.2-q12.2in decreasing order of significance.

The 8q24.21, 11q13.3 and 3q11.2-q12.2 regions have all previously been reported in a variety of carcinomas[51-53] and they often co-exist with one another. Genomic amplification at 8q24 occurs in a large variety of cancers[51,53-56], and most amplicons described in the literature involve both the *C-MYC* and *FAM84B* genes, as was observed in this study in four ESCC cell lines. In previous reports the target of amplification has been attributed to either both genes[54], or to one or the other[55,56] based on their respective increased transcription.

The 11q13.3 amplicon covered a large region containing a number of potential target genes. The *CCND1* and *MYEOV* genes (11q13.3) were co-amplified in four cell

lines. Co-amplification of these genes has been reported in multiple myeloma, breast cancer and ESCC[14]. *CCND1* is a downstream effector in the Wnt2/β-catenin pathway and the most frequent target of amplification in several ESCC studies[14,53,57]. While the *MYEOV* gene has been associated with cell proliferation in colon cancer[58], its amplification in ESCC is not always matched by increased transcription due to its silencing by epigenetic mechanisms[59]. The cortactin gene, *CTTN,* involved in cell motility[60], was previously shown to be over expressed in ESCC pre-cancerous lesions, as well as in carcinogen induced murine ESCC supporting a role for this gene in ESCC carcinogenesis[61]. The three fibroblast growth factor (FGF) genes, *FGF3*, *FGF4* and *FGF19* were part of the 11q13.3 amplicon. FGF and Wnt signaling pathways cross talk in a number of carcinogenesis scenarios[62]. Activated FGF receptors activate the FRS-GR2-GAB1-PI3K-AKT signaling cascade, and downregulate GSK-3β protein activity, thus hampering β-catenin phosphorylation and degradation[62]. In particular, FGF19 ligand downregulates GSK-3β activity, which results in the release and nuclear accumulation of β-catenin. Nuclear β-catenin activates the transcription of downstream genes including *C-MYC* and *CCND1*[63].

In addition to the amplification of Wnt pathway activators, the *SFRP2* tumor suppressor gene locus was deleted at chromosome 4q22.1-q32.1 (Figure 7). The *SFRP2* gene encodes a frizzled-related protein and is part of the SFRP family of Wnt inhibitors. Loss of *SFRP2* is detected in medulloblastoma and is suggested to contribute to carcinogenesis through loss of inhibition of the Wnt pathway[64].

The copy number data therefore suggests that the Wnt signaling pathway may be at work in these ESCC cell lines through one or the combined effects of genes activating the β-catenin transcriptional activity and/or the FGF signaling pathways as well as deletions of genes, at 4q22.1-q32.1, inhibiting this pathway. Amplicons at both 8q24 and 11q13.3-13.4 have been described in a variety of squamous cell carcinoma[54,65-67] suggesting that the activation of pathways through the combined effects of genes at 8q24 and 11q13.3-13.4 contributes to the development and aggressiveness of SCC.

In addition to the *SFRP2* gene, the three tumor suppressor genes *BMR1B, SMAD1* and *CASP6* were also targets of deletion at 4q22.1-q32.1. Both *BMR1B* and *SMAD1* genes have previously been reported to have decreased expression in gliomas correlating with poor survival[68], and *BMPR1B* decreased expression in breast cancer is associated with increased cell proliferation and poor prognosis[69]. The *CASP6* gene encodes the pro-apoptotic caspase-6 protein[70].

Although large 3q amplicons are commonly observed in squamous carcinoma[71,72] in this study the 6 Mb, 3q11.2-12.2 amplicon was focal and involved the *MINA* gene. This gene has previously been reported to be over-expressed in 83% of ESCC in one study and its inhibition was shown to suppress ESCC cell proliferation[73].

A 43 Mb region of deletion at 8p23.2-q11.1 (4 078 057-47 043 375 bp) was observed in the five cell lines and involved five potential target genes. These included the *BNIP3L* gene deleted or downregulated in prostate cancer and malignant melanomas, respectively[65,74], the *LZTS1* gene, deleted in oral squamous cell carcinomas and downregulated in breast carcinomas[75,76]; and the three *TNFR* genes, *TNFRSF10A*, *TNFRSF10B* and *TNFRSF10C,* whose epigenetic inactivation was reported in gastric cancers[77]. An 8p loss, was previously detected by conventional CGH in a study performed on 29 South African black and colored ESCC patients[78]. Chromosome 8p22 loss has also been reported in prostate, breast, lymphoma, hepatocellular and colorectal cancers[79-83].

Active smoking is linked to increased fragile site expression[84] and is also one of the primary risk factors associated with ESCC in South Africa[85]. We previously hypothesized that deletions affecting anti-oncogenes located at fragile sites may contribute to the etiology of ESCC in South Africa and reported *FHIT* intragenic deletions in these cell lines and a small cohort of patients[40]. *FHIT* gene deletions were confirmed here supporting its role in ESCC carcinogenesis.

Deletion at 18q23 involved the *SMAD4* and *DCC* genes in all cell lines. Both genes have previously been reported to be down regulated in ESCC either by deletion, mutation or methylation[86]. Decreased expression of the *SMAD4* gene, a tumor suppressor of the transforming growth factor β family signaling pathway, has been associated with ESCC tumor invasion[87]. The *DCC* gene was shown to be frequently methylated in ESCC tumor specimens[88].

In summary, breakpoints at 1p11 and 3p11 were recurrent in the five ESCC cell lines and may point to genes such as *EPHA3* that may be involved in ESCC carcinogenesis. Copy number alterations involved both amplicons previously reported in squamous cell carcinoma (8q24, 11q13 and 3q11) as well as novel regions of significant amplification (11q22.1-q22.3, 5p15.2 and 18p11.32). The finding that a significant number of genes that were amplified (*FGF3, FGF4, FGF19, CCND* and *C-MYC)* or deleted (*SFRP2* gene) are involved in the Wnt and FGF signaling pathways suggest that these pathways may be activated in these ESCC cell lines. These results warrant expression studies of these genes in both cell lines and patients' specimens. Of interest, should *FGF* gene expression be increased, ESCC patients may benefit from the respective FGF targeted therapies recently developed[89,90].

ACKNOWLEDGMENTS

We thank Elsabe Scott for culturing the cell lines.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is a major cause of cancer death in the world and has a peculiar epidemiology with worldwide geographic

pockets of high incidence particularly in Asia and Africa. In South Africa, in the Eastern Cape region, ESCC represents the leading cancer affecting men and the second most common cancer in woman. There is a need to identify biomarkers to better understand the pathophysiology of this cancer and inform future diagnostic and therapeutic strategies for these patients. Established cell lines provide a unique resource to investigate both the presence of chromosomes translocations and copy number imbalances.

Research frontiers

A limited number of ESCC cell lines, all established in Asia, were reported to date. These have been investigated with molecular cytogenetic techniques of limited resolution and no key pathways have been reported previously.

Innovations and breakthroughs

This is the first comprehensive molecular cytogenetic study of five ESCC cell lines established in South Africa. The authors combined high-resolution whole genome array copy number analysis with conventional cytogenetics and multicolor fluorescence *in situ* hybridization to assess common chromosomal imbalances. Interestingly, a significant number of genes that were amplified (*FGF3*, *FGF4*, *FGF19*, *CCND1* and *C-MYC*) or deleted (*SFRP2* gene) are involved in the Wnt and FGF signaling pathways. In addition, a deletion within or near the *EPHA3* gene was present in 4 of these cell lines, corresponding to a translocation breakpoint at 3p11.2 shared in some cell lines.

Applications

These results suggest that the Wnt and FGF pathways may be involved in the initiation or progression of ESCC. They also point to the *EPHA3* gene as an added potential key gene. Further study on patients' specimens and functional studies will determine the significance of these genes in ESCC pathogenesis.

Terminology

High-resolution 250K single nucleotide polymorphism (SNP) arrays cover at least one SNP per 100 kb of DNA, using an average of 24 probes per SNP. The copy number data are derived from the summary of non-polymorphic SNPs and examined as a ratio to a reference genome. Changes in intensity ratios are indicative of amplification/deletions. The Wnt and fibroblast growth factor (FGF) signaling pathways are involved in tissue homeostasis as well as in cell proliferation and differentiation. Although their mechanisms differ, these two pathways cross talk through GSK3β inhibition.

Peer review

The manuscript by Brown and coworkers demonstrates that a significant number of genes that were amplified or deleted are involved in the Wnt and FGF signaling pathways in five cell lines established from South African ESCC patients. They suggest that these pathways are activated in these cell lines. The overall goal of the paper is relevant. The data presented are solid and credible. The results are interesting and clinically important.

REFERENCES

1 **Somdyala NI**, Bradshaw D, Gelderblom WC, Parkin DM. Cancer incidence in a rural population of South Africa, 1998-2002. *Int J Cancer* 2010; **127**: 2420-2429

2 **Hendricks D**, Parker MI. Oesophageal cancer in Africa. *IUBMB Life* 2002; **53**: 263-268

3 **van Rensburg SJ**. Oesophageal cancer risk factors common to endemic regions. *S Afr Med J* 1987; **Suppl**: 9-11

4 **Kamangar F**, Malekzadeh R, Dawsey SM, Saidi F. Esophageal cancer in Northeastern Iran: a review. *Arch Iran Med* 2007; **10**: 70-82

5 **Marasas WF**, van Rensburg SJ, Mirocha CJ. Incidence of Fusarium species and the mycotoxins, deoxynivalenol and zearalenone, in corn produced in esophageal cancer areas in Transkei. *J Agric Food Chem* 1979; **27**: 1108-1112

6 **Sun G**, Wang S, Hu X, Su J, Huang T, Yu J, Tang L, Gao W, Wang JS. Fumonisin B1 contamination of home-grown corn in high-risk areas for esophageal and liver cancer in China. *Food Addit Contam* 2007; **24**: 181-185

7 **Yao PF**, Li GC, Li J, Xia HS, Yang XL, Huang HY, Fu YG, Wang RQ, Wang XY, Sha JW. Evidence of human papilloma virus infection and its epidemiology in esophageal squamous cell carcinoma. *World J Gastroenterol* 2006; **12**: 1352-1355

8 **Matsha T**, Stepien A, Blanco-Blanco E, Brink LT, Lombard CJ, Van Rensburg S, Erasmus RT. Self-induced vomiting -- risk for oesophageal cancer? *S Afr Med J* 2006; **96**: 209-212

9 **Mitelman F**, Johansson B, Mertens F. The impact of translocations and gene fusions on cancer causation. *Nat Rev Cancer* 2007; **7**: 233-245

10 **Hu YC**, Lam KY, Law SY, Wan TS, Ma ES, Kwong YL, Chan LC, Wong J, Srivastava G. Establishment, characterization, karyotyping, and comparative genomic hybridization analysis of HKESC-2 and HKESC-3: two newly established human esophageal squamous cell carcinoma cell lines. *Cancer Genet Cytogenet* 2002; **135**: 120-127

11 **Wu YP**, Yang YL, Yang GZ, Wang XY, Luo ML, Zhang Y, Feng YB, Xu X, Han YL, Cai Y, Zhan QM, Wu M, Dong JT, Wang MR. Identification of chromosome aberrations in esophageal cancer cell line KYSE180 by multicolor fluorescence in situ hybridization. *Cancer Genet Cytogenet* 2006; **170**: 102-107

12 **Xiao S**, Feng XL, Geng JS, Yan FC, Liu QZ, Li P. Cytogenetic studies of five primary esophageal cancers. *Cancer Genet Cytogenet* 1991; **55**: 197-205

13 **Tada K**, Oka M, Hayashi H, Tangoku A, Oga A, Sasaki K. Cytogenetic analysis of esophageal squamous cell carcinoma cell lines by comparative genomic hybridization: relationship of cytogenetic aberrations to in vitro cell growth. *Cancer Genet Cytogenet* 2000; **117**: 108-112

14 **Jin Y**, Jin C, Law S, Chu KM, Zhang H, Strombeck B, Yuen AP, Kwong YL. Cytogenetic and fluorescence in situ hybridization characterization of clonal chromosomal aberrations and CCND1 amplification in esophageal carcinomas. *Cancer Genet Cytogenet* 2004; **148**: 21-28

15 **Bey E**, Alexander J, Whitcutt JM, Hunt JA, Gear JH. Carcinoma of the esophagus in Africans: establishment of a continuously growing cell line from a tumor specimen. *In Vitro* 1976; **12**: 107-114

16 **Veale RB**, Thornley AL. Increased single class low-affinity EGF receptors expressed by human oesophageal squamous carcinoma cell lines. *S Afr J Sci* 1989; **85**: 375-379

17 **Reich M**, Liefeld T, Gould J, Lerner J, Tamayo P, Mesirov JP. GenePattern 2.0. *Nat Genet* 2006; **38**: 500-501

18 **Hupé P**, Stransky N, Thiery JP, Radvanyi F, Barillot E. Analysis of array CGH data: from signal ratio to gain and loss of DNA regions. *Bioinformatics* 2004; **20**: 3413-3422

19 **Beroukhim R**, Getz G, Nghiemphu L, Barretina J, Hsueh T, Linhart D, Vivanco I, Lee JC, Huang JH, Alexander S, Du J, Kau T, Thomas RK, Shah K, Soto H, Perner S, Prensner J, Debiasi RM, Demichelis F, Hatton C, Rubin MA, Garraway LA, Nelson SF, Liau L, Mischel PS, Cloughesy TF, Meyerson M, Golub TA, Lander ES, Mellinghoff IK, Sellers WR. Assessing the significance of chromosomal aberrations in cancer: methodology and application to glioma. *Proc Natl Acad Sci USA* 2007; **104**: 20007-20012

20 **Haverty PM**, Fridlyand J, Li L, Getz G, Beroukhim R, Lohr S, Wu TD, Cavet G, Zhang Z, Chant J. High-resolution genomic and expression analyses of copy number alterations in breast tumors. *Genes Chromosomes Cancer* 2008; **47**: 530-542

21 **Weir BA**, Woo MS, Getz G, Perner S, Ding L, Beroukhim R, Lin WM, Province MA, Kraja A, Johnson LA, Shah K, Sato M, Thomas RK, Barletta JA, Borecki IB, Broderick S, Chang AC, Chiang DY, Chirieac LR, Cho J, Fujii Y, Gazdar AF, Giordano T, Greulich H, Hanna M, Johnson BE, Kris MG, Lash A, Lin L, Lindeman N, Mardis ER, McPherson JD, Minna JD, Morgan MB, Nadel M, Orringer MB, Osborne JR, Ozenberger B, Ramos AH, Robinson J, Roth JA, Rusch V, Sasaki H, Shepherd F, Sougnez C, Spitz MR, Tsao MS, Twomey D, Verhaak RG, Weinstock GM, Wheeler DA, Winckler W, Yoshizawa A, Yu S, Zakowski MF, Zhang Q, Beer DG, Wistuba II, Watson MA, Garraway LA, Ladanyi M, Travis WD, Pao W, Rubin MA, Gabriel SB, Gibbs RA, Varmus HE, Wilson RK, Lander ES, Meyerson M. Characterizing the cancer genome in lung adenocarcinoma. *Nature* 2007; **450**: 893-898

22 **Davies H**, Hunter C, Smith R, Stephens P, Greenman C, Bignell G, Teague J, Butler A, Edkins S, Stevens C, Parker A, O'Meara S, Avis T, Barthorpe S, Brackenbury L, Buck G, Clements J, Cole J, Dicks E, Edwards K, Forbes S, Gorton M, Gray K, Halliday K, Harrison R, Hills K, Hinton J, Jones D, Kosmidou V, Laman R, Lugg R, Menzies A, Perry J, Petty R, Raine K, Shepherd R, Small A, Solomon H, Stephens Y, Tofts C, Varian J, Webb A, West S, Widaa S, Yates A, Brasseur F, Cooper CS, Flanagan AM, Green A, Knowles M, Leung SY, Looijenga LH, Malkowicz B, Pierotti MA, Teh BT, Yuen ST, Lakhani SR, Easton DF, Weber BL, Goldstraw P, Nicholson AG, Wooster R, Stratton MR, Futreal PA. Somatic mutations of the protein kinase gene family in human lung cancer. *Cancer Res* 2005; **65**: 7591-7595

23 **Stephens P**, Edkins S, Davies H, Greenman C, Cox C, Hunter C, Bignell G, Teague J, Smith R, Stevens C, O'Meara S, Parker A, Tarpey P, Avis T, Barthorpe A, Brackenbury L, Buck G, Butler A, Clements J, Cole J, Dicks E, Edwards K, Forbes S, Gorton M, Gray K, Halliday K, Harrison R, Hills K, Hinton J, Jones D, Kosmidou V, Laman R, Lugg R, Menzies A, Perry J, Petty R, Raine K, Shepherd R, Small A, Solomon H, Stephens Y, Tofts C, Varian J, Webb A, West S, Widaa S, Yates A, Brasseur F, Cooper CS, Flanagan AM, Green A, Knowles M, Leung SY, Looijenga LH, Malkowicz B, Pierotti MA, Teh B, Yuen ST, Nicholson AG, Lakhani S, Easton DF, Weber BL, Stratton MR, Futreal PA, Wooster R. A screen of the complete protein kinase gene family identifies diverse patterns of somatic mutations in human breast cancer. *Nat Genet* 2005; **37**: 590-592

24 **Wood LD**, Calhoun ES, Silliman N, Ptak J, Szabo S, Powell SM, Riggins GJ, Wang TL, Yan H, Gazdar A, Kern SE, Pennacchio L, Kinzler KW, Vogelstein B, Velculescu VE. Somatic mutations of GUCY2F, EPHA3, and NTRK3 in human cancers. *Hum Mutat* 2006; **27**: 1060-1061

25 **Puca R**, Nardinocchi L, Sacchi A, Rechavi G, Givol D, D'Orazi G. HIPK2 modulates p53 activity towards pro-apoptotic transcription. *Mol Cancer* 2009; **8**: 85

26 **Imoto I**, Tsuda H, Hirasawa A, Miura M, Sakamoto M, Hirohashi S, Inazawa J. Expression of cIAP1, a target for 11q22 amplification, correlates with resistance of cervical cancers to radiotherapy. *Cancer Res* 2002; **62**: 4860-4866

27 **Da CL**, Xin Y, Zhao J, Luo XD. Significance and relationship between Yes-associated protein and survivin expression in gastric carcinoma and precancerous lesions. *World J Gastroenterol* 2009; **15**: 4055-4061

28 **Gu ZD**, Li JY, Li M, Gu J, Shi XT, Ke Y, Chen KN. Matrix metalloproteinases expression correlates with survival in patients with esophageal squamous cell carcinoma. *Am J Gastroenterol* 2005; **100**: 1835-1843

29 **Burger MJ**, Tebay MA, Keith PA, Samaratunga HM, Clements J, Lavin MF, Gardiner RA. Expression analysis of delta-catenin and prostate-specific membrane antigen: their potential as diagnostic markers for prostate cancer. *Int J Cancer* 2002; **100**: 228-237

30 **Or YY**, Hui AB, Tam KY, Huang DP, Lo KW. Characterization of chromosome 3q and 12q amplicons in nasopharyngeal carcinoma cell lines. *Int J Oncol* 2005; **26**: 49-56

31 **Chen J**, Guo L, Peiffer DA, Zhou L, Chan OT, Bibikova M, Wickham-Garcia E, Lu SH, Zhan Q, Wang-Rodriguez J, Jiang W, Fan JB. Genomic profiling of 766 cancer-related genes in archived esophageal normal and carcinoma tissues. *Int J Cancer* 2008; **122**: 2249-2254

32 **Nakakuki K**, Imoto I, Pimkhaokham A, Fukuda Y, Shimada Y, Imamura M, Amagasa T, Inazawa J. Novel targets for the 18p11.3 amplification frequently observed in esophageal squamous cell carcinomas. *Carcinogenesis* 2002; **23**: 19-24

33 **Jensen SA**, Vainer B, Witton CJ, Jørgensen JT, Sørensen JB. Prognostic significance of numeric aberrations of genes for thymidylate synthase, thymidine phosphorylase and dihydrofolate reductase in colorectal cancer. *Acta Oncol* 2008; **47**: 1054-1061

34 **Huang J**, Lin Y, Li L, Qing D, Teng XM, Zhang YL, Hu X, Hu Y, Yang P, Han ZG. ARHI, as a novel suppressor of cell growth and downregulated in human hepatocellular carcinoma, could contribute to hepatocarcinogenesis. *Mol Carcinog* 2009; **48**: 130-140

35 **Shi Z**, Zhou X, Xu L, Zhang T, Hou Y, Zhu W, Zhang T. [NOEY2 gene mRNA expression in breast cancer tissue and its relation to clinicopathological parameters]. *Zhonghua Zhongliu Zazhi* 2002; **24**: 475-478

36 **Rosemary Siafakas A**, Richardson DR. Growth arrest and DNA damage-45 alpha (GADD45alpha). *Int J Biochem Cell Biol* 2009; **41**: 986-989

37 **Yang G**, Cao K, Wu L, Wang R. Cystathionine gamma-lyase overexpression inhibits cell proliferation via a H2S-dependent modulation of ERK1/2 phosphorylation and p21Cip/WAK-1. *J Biol Chem* 2004; **279**: 49199-49205

38 **Liu CX**, Ranganathan S, Robinson S, Strickland DK. gamma-Secretase-mediated release of the low density lipoprotein receptor-related protein 1B intracellular domain suppresses anchorage-independent growth of neuroglioma cells. *J Biol Chem* 2007; **282**: 7504-7511

39 **Nagayama K**, Kohno T, Sato M, Arai Y, Minna JD, Yokota J. Homozygous deletion scanning of the lung cancer genome at a 100-kb resolution. *Genes Chromosomes Cancer* 2007; **46**: 1000-1010

40 **Willem P**, Brown J, Schouten J. A novel approach to simultaneously scan genes at fragile sites. *BMC Cancer* 2006; **6**: 205

41 **Lo PH**, Lung HL, Cheung AK, Apte SS, Chan KW, Kwong FM, Ko JM, Cheng Y, Law S, Srivastava G, Zabarovsky ER, Tsao SW, Tang JC, Stanbridge EJ, Lung ML. Extracellular protease ADAMTS9 suppresses esophageal and nasopharyngeal carcinoma tumor formation by inhibiting angiogenesis. *Cancer Res* 2010; **70**: 5567-5576

42 **Cheung LC**, Tang JC, Lee PY, Hu L, Guan XY, Tang WK, Srivastava G, Wong J, Luk JM, Law S. Establishment and characterization of a new xenograft-derived human esophageal squamous cell carcinoma cell line HKESC-4 of Chinese origin. *Cancer Genet Cytogenet* 2007; **178**: 17-25

43 **Yang Y**, Chu J, Wu Y, Luo M, Xu X, Han Y, Cai Y, Zhan Q, Wang M. Chromosome analysis of esophageal squamous cell carcinoma cell line KYSE 410-4 by repetitive multicolor fluorescence in situ hybridization. *J Genet Genomics* 2008; **35**: 11-16

44 **Yen CC**, Chen YJ, Chen JT, Hsia JY, Chen PM, Liu JH, Fan FS, Chiou TJ, Wang WS, Lin CH. Comparative genomic hybridization of esophageal squamous cell carcinoma: correlations between chromosomal aberrations and disease progression/prognosis. *Cancer* 2001; **92**: 2769-2777

45 **Jin Y**, Jin C, Salemark L, Martins C, Wennerberg J, Mertens F. Centromere cleavage is a mechanism underlying isochromosome formation in skin and head and neck carcinomas. *Chromosoma* 2000; **109**: 476-481

46 **Johansson B**, Mertens F. Frequency and distribution of mitomycin C-induced structural chromosome aberrations in lymphocytes from non-Hodgkin lymphoma patients. *Cytogenet Cell Genet* 1988; **48**: 79-83

47 **Kleinsasser NH**, Sassen AW, Semmler MP, Staudenmaier R, Harréus UA, Richter E. [Does nicotine add to the carcinogenic strain of tobacco smoke?]. *HNO* 2006; **54**: 369-372, 374-375

48 **Stein CK**, Glover TW, Palmer JL, Glisson BS. Direct correlation between FRA3B expression and cigarette smoking. *Genes Chromosomes Cancer* 2002; **34**: 333-340

49 **Clifford N**, Smith LM, Powell J, Gattenlöhner S, Marx A, O'Connor R. The EphA3 receptor is expressed in a subset of rhabdomyosarcoma cell lines and suppresses cell adhesion and migration. *J Cell Biochem* 2008; **105**: 1250-1259

50 **Zhang H**, Jin Y, Chen X, Jin C, Law S, Tsao SW, Kwong YL. Cytogenetic aberrations in immortalization of esophageal epithelial cells. *Cancer Genet Cytogenet* 2006; **165**: 25-35

51 **Burkhardt L**, Grob TJ, Hermann I, Burandt E, Choschzick M,

Jänicke F, Müller V, Bokemeyer C, Simon R, Sauter G, Wilczak W, Lebeau A. Gene amplification in ductal carcinoma in situ of the breast. *Breast Cancer Res Treat* 2010; **123**: 757-765

52 **Freier K**, Joos S, Flechtenmacher C, Devens F, Benner A, Bosch FX, Lichter P, Hofele C. Tissue microarray analysis reveals site-specific prevalence of oncogene amplifications in head and neck squamous cell carcinoma. *Cancer Res* 2003; **63**: 1179-1182

53 **Bass AJ**, Watanabe H, Mermel CH, Yu S, Perner S, Verhaak RG, Kim SY, Wardwell L, Tamayo P, Gat-Viks I, Ramos AH, Woo MS, Weir BA, Getz G, Beroukhim R, O'Kelly M, Dutt A, Rozenblatt-Rosen O, Dziunycz P, Komisarof J, Chirieac LR, Lafargue CJ, Scheble V, Wilbertz T, Ma C, Rao S, Nakagawa H, Stairs DB, Lin L, Giordano TJ, Wagner P, Minna JD, Gazdar AF, Zhu CQ, Brose MS, Cecconello I, Jr UR, Marie SK, Dahl O, Shivdasani RA, Tsao MS, Rubin MA, Wong KK, Regev A, Hahn WC, Beer DG, Rustgi AK, Meyerson M. SOX2 is an amplified lineage-survival oncogene in lung and esophageal squamous cell carcinomas. *Nat Genet* 2009; **41**: 1238-1242

54 **Camps J**, Nguyen QT, Padilla-Nash HM, Knutsen T, McNeil NE, Wangsa D, Hummon AB, Grade M, Ried T, Difilippantonio MJ. Integrative genomics reveals mechanisms of copy number alterations responsible for transcriptional deregulation in colorectal cancer. *Genes Chromosomes Cancer* 2009; **48**: 1002-1017

55 **Huang XP**, Rong TH, Wang JY, Tang YQ, Li BJ, Xu DR, Zhao MQ, Zhang LJ, Fang Y, Su XD, Liang QW. Negative implication of C-MYC as an amplification target in esophageal cancer. *Cancer Genet Cytogenet* 2006; **165**: 20-24

56 **van Duin M**, van Marion R, Vissers KJ, Hop WC, Dinjens WN, Tilanus HW, Siersema PD, van Dekken H. High-resolution array comparative genomic hybridization of chromosome 8q: evaluation of putative progression markers for gastroesophageal junction adenocarcinomas. *Cytogenet Genome Res* 2007; **118**: 130-137

57 **Janssen JW**, Cuny M, Orsetti B, Rodriguez C, Vallés H, Bartram CR, Schuuring E, Theillet C. MYEOV: a candidate gene for DNA amplification events occurring centromeric to CCND1 in breast cancer. *Int J Cancer* 2002; **102**: 608-614

58 **Moss AC**, Lawlor G, Murray D, Tighe D, Madden SF, Mulligan AM, Keane CO, Brady HR, Doran PP, MacMathuna P. ETV4 and Myeov knockdown impairs colon cancer cell line proliferation and invasion. *Biochem Biophys Res Commun* 2006; **345**: 216-221

59 **Janssen JW**, Imoto I, Inoue J, Shimada Y, Ueda M, Imamura M, Bartram CR, Inazawa J. MYEOV, a gene at 11q13, is coamplified with CCND1, but epigenetically inactivated in a subset of esophageal squamous cell carcinomas. *J Hum Genet* 2002; **47**: 460-464

60 **Hofman P**, Butori C, Havet K, Hofman V, Selva E, Guevara N, Santini J, Van Obberghen-Schilling E. Prognostic significance of cortactin levels in head and neck squamous cell carcinoma: comparison with epidermal growth factor receptor status. *Br J Cancer* 2008; **98**: 956-964

61 **Hsu NY**, Yeh KT, Chiang IP, Pai LY, Chen CY, Ho HC. Cortactin overexpression in the esophageal squamous cell carcinoma and its involvement in the carcinogenesis. *Dis Esophagus* 2008; **21**: 402-408

62 **Katoh M**, Katoh M. Cross-talk of WNT and FGF signaling pathways at GSK3beta to regulate beta-catenin and SNAIL signaling cascades. *Cancer Biol Ther* 2006; **5**: 1059-1064

63 **Novak A**, Dedhar S. Signaling through beta-catenin and Lef/Tcf. *Cell Mol Life Sci* 1999; **56**: 523-537

64 **Kongkham PN**, Northcott PA, Croul SE, Smith CA, Taylor MD, Rutka JT. The SFRP family of WNT inhibitors function as novel tumor suppressor genes epigenetically silenced in medulloblastoma. *Oncogene* 2010; **29**: 3017-3024

65 **Liu W**, Xie CC, Zhu Y, Li T, Sun J, Cheng Y, Ewing CM, Dalrymple S, Turner AR, Sun J, Isaacs JT, Chang BL, Zheng SL, Isaacs WB, Xu J. Homozygous deletions and recurrent amplifications implicate new genes involved in prostate cancer. *Neoplasia* 2008; **10**: 897-907

66 **Janssen JW**, Vaandrager JW, Heuser T, Jauch A, Kluin PM, Geelen E, Bergsagel PL, Kuehl WM, Drexler HG, Otsuki T, Bartram CR, Schuuring E. Concurrent activation of a novel putative transforming gene, myeov, and cyclin D1 in a subset of multiple myeloma cell lines with t(11; 14)(q13; q32). *Blood* 2000; **95**: 2691-2698

67 **Fantozzi I**, Grall D, Cagnol S, Stanchi F, Sudaka A, Brunstein MC, Bozec A, Fischel JL, Milano G, Van Obberghen-Schilling E. Overexpression of cortactin in head and neck squamous cell carcinomas can be uncoupled from augmented EGF receptor expression. *Acta Oncol* 2008; **47**: 1502-1512

68 **Liu S**, Tian Z, Yin F, Zhang P, W Y, Ding X, Wu H, Wu Y, Peng X, Yuan J, Qiang B, Fan W, Fan M. Expression and functional roles of Smad1 and BMPR-IB in glioma development. *Cancer Invest* 2009; **27**: 734-740

69 **Bokobza SM**, Ye L, Kynaston HE, Mansel RE, Jiang WG. Reduced expression of BMPR-IB correlates with poor prognosis and increased proliferation of breast cancer cells. *Cancer Genomics Proteomics* 2009; **6**: 101-108

70 **Chan JY**, Phoo MS, Clement MV, Pervaiz S, Lee SC. Resveratrol displays converse dose-related effects on 5-fluorouracil-evoked colon cancer cell apoptosis: the roles of caspase-6 and p53. *Cancer Biol Ther* 2008; **7**: 1305-1312

71 **Haverty PM**, Hon LS, Kaminker JS, Chant J, Zhang Z. High-resolution analysis of copy number alterations and associated expression changes in ovarian tumors. *BMC Med Genomics* 2009; **2**: 21

72 **Kanao H**, Enomoto T, Kimura T, Fujita M, Nakashima R, Ueda Y, Ueno Y, Miyatake T, Yoshizaki T, Buzard GS, Tanigami A, Yoshino K, Murata Y. Overexpression of LAMP3/TSC403/DC-LAMP promotes metastasis in uterine cervical cancer. *Cancer Res* 2005; **65**: 8640-8645

73 **Tsuneoka M**, Fujita H, Arima N, Teye K, Okamura T, Inutsuka H, Koda Y, Shirouzu K, Kimura H. Mina53 as a potential prognostic factor for esophageal squamous cell carcinoma. *Clin Cancer Res* 2004; **10**: 7347-7356

74 **Su DM**, Zhang Q, Wang X, He P, Zhu YJ, Zhao J, Rennert OM, Su YA. Two types of human malignant melanoma cell lines revealed by expression patterns of mitochondrial and survival-apoptosis genes: implications for malignant melanoma therapy. *Mol Cancer Ther* 2009; **8**: 1292-1304

75 **Chen L**, Zhu Z, Sun X, Dong XY, Wei J, Gu F, Sun YL, Zhou J, Dong JT, Fu L. Down-regulation of tumor suppressor gene FEZ1/LZTS1 in breast carcinoma involves promoter methylation and associates with metastasis. *Breast Cancer Res Treat* 2009; **116**: 471-478

76 **Ono K**, Uzawa K, Nakatsuru M, Shiiba M, Mochida Y, Tada A, Bukawa H, Miyakawa A, Yokoe H, Tanzawa H. Down-regulation of FEZ1/LZTS1 gene with frequent loss of heterozygosity in oral squamous cell carcinomas. *Int J Oncol* 2003; **23**: 297-302

77 **Lee KH**, Lim SW, Kim HG, Kim DY, Ryu SY, Joo JK, Kim JC, Lee JH. Lack of death receptor 4 (DR4) expression through gene promoter methylation in gastric carcinoma. *Langenbecks Arch Surg* 2009; **394**: 661-670

78 **Du Plessis L**, Dietzsch E, Van Gele M, Van Roy N, Van Helden P, Parker MI, Mugwanya DK, De Groot M, Marx MP, Kotze MJ, Speleman F. Mapping of novel regions of DNA gain and loss by comparative genomic hybridization in esophageal carcinoma in the Black and Colored populations of South Africa. *Cancer Res* 1999; **59**: 1877-1883

79 **Bova GS**, MacGrogan D, Levy A, Pin SS, Bookstein R, Isaacs WB. Physical mapping of chromosome 8p22 markers and their homozygous deletion in a metastatic prostate cancer. *Genomics* 1996; **35**: 46-54

80 **Di Benedetto M**, Pineau P, Nouet S, Berhouet S, Seitz I, Louis S, Dejean A, Couraud PO, Strosberg AD, Stoppa-Lyonnet D, Nahmias C. Mutation analysis of the 8p22 candidate tumor

suppressor gene ATIP/MTUS1 in hepatocellular carcinoma. *Mol Cell Endocrinol* 2006; **252**: 207-215

81 **Flanagan JM**, Healey S, Young J, Whitehall V, Trott DA, Newbold RF, Chenevix-Trench G. Mapping of a candidate colorectal cancer tumor-suppressor gene to a 900-kilobase region on the short arm of chromosome 8. *Genes Chromosomes Cancer* 2004; **40**: 247-260

82 **Thomassen M**, Jochumsen KM, Mogensen O, Tan Q, Kruse TA. Gene expression meta-analysis identifies chromosomal regions involved in ovarian cancer survival. *Genes Chromosomes Cancer* 2009; **48**: 711-724

83 **Ying J**, Li H, Murray P, Gao Z, Chen YW, Wang Y, Lee KY, Chan AT, Ambinder RF, Srivastava G, Tao Q. Tumor-specific methylation of the 8p22 tumor suppressor gene DLC1 is an epigenetic biomarker for Hodgkin, nasal NK/T-cell and other types of lymphomas. *Epigenetics* 2007; **2**: 15-21

84 **Sozzi G**, Sard L, De Gregorio L, Marchetti A, Musso K, Buttitta F, Tornielli S, Pellegrini S, Veronese ML, Manenti G, Incarbone M, Chella A, Angeletti CA, Pastorino U, Huebner K, Bevilaqua G, Pilotti S, Croce CM, Pierotti MA. Association between cigarette smoking and FHIT gene alterations in lung cancer. *Cancer Res* 1997; **57**: 2121-2123

85 **Pacella-Norman R**, Urban MI, Sitas F, Carrara H, Sur R, Hale M, Ruff P, Patel M, Newton R, Bull D, Beral V. Risk factors for oesophageal, lung, oral and laryngeal cancers in black South Africans. *Br J Cancer* 2002; **86**: 1751-1756

86 **Metzger R**, Schneider PM, Warnecke-Eberz U, Brabender J, Hölscher AH. Molecular biology of esophageal cancer. *Onkologie* 2004; **27**: 200-206

87 **Fukuchi M**, Masuda N, Miyazaki T, Nakajima M, Osawa H, Kato H, Kuwano H. Decreased Smad4 expression in the transforming growth factor-beta signaling pathway during progression of esophageal squamous cell carcinoma. *Cancer* 2002; **95**: 737-743

88 **Park HL**, Kim MS, Yamashita K, Westra W, Carvalho AL, Lee J, Jiang WW, Baek JH, Liu J, Osada M, Moon CS, Califano JA, Mori M, Sidransky D. DCC promoter hypermethylation in esophageal squamous cell carcinoma. *Int J Cancer* 2008; **122**: 2498-2502

89 **Pai R**, Dunlap D, Qing J, Mohtashemi I, Hotzel K, French DM. Inhibition of fibroblast growth factor 19 reduces tumor growth by modulating beta-catenin signaling. *Cancer Res* 2008; **68**: 5086-5095

90 **Stemke-Hale K**, Gonzalez-Angulo AM, Lluch A, Neve RM, Kuo WL, Davies M, Carey M, Hu Z, Guan Y, Sahin A, Symmans WF, Pusztai L, Nolden LK, Horlings H, Berns K, Hung MC, van de Vijver MJ, Valero V, Gray JW, Bernards R, Mills GB, Hennessy BT. An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer. *Cancer Res* 2008; **68**: 6084-6091

S- Editor Tian L **L- Editor** Cant MR **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2924

World J Gastroenterol 2011 June 28; 17(24): 2924-2932
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2011 Baishideng. All rights reserved.

ORIGINAL ARTICLE

Characterization of a novel rat cholangiocarcinoma cell culture model-CGCCA

Chun-Nan Yeh, Kun-Ju Lin, Tsung-Wen Chen, Ren-Ching Wu, Lee-Cheng Tsao, Ying-Tzu Chen, Wen-Hui Weng, Miin-Fu Chen

Chun-Nan Yeh, Tsung-Wen Chen, Miin-Fu Chen, Department of Surgery, Chang Gung Memorial Hospital, Chang Gung University, Taoyuan 333, Taiwan, China
Kun-Ju Lin, Department of Nuclear Medicine; Chang Gung Memorial Hospital, Chang Gung University, Taoyuan 333, Taiwan, China
Ren-Ching Wu, Department of Pathology, Chang Gung Memorial Hospital, Chang Gung University, Taoyuan 333, Taiwan, China
Lee-Cheng Tsao, Ying-Tzu Chen, Wen-Hui Weng, Graduate Institute of Biotechnology and Department of Chemical Engineering and Biotechnology, National Taipei University of Technology, Taipei 106, Taiwan, China
Author contributions: Yeh CN helped collect the data and wrote the manuscript; Yeh CN and Weng WH were in charge of this project and revised the manuscript; Lin KJ was in charge of the FDG-PET; Chen TW was in charge of cell-line establishment; Wu RC was in charge of IHC study; Tsao LC and Chen YT were in charge of cytogenetic study; Chen MF helped to review this paper.
Supported by Chang Gung Medical Research, Program grant 350363, National Science Council grants to Yeh CN; National Science Council grant 98-2314-B-027-001 to Weng WH
Correspondence to: Wen-Hui Weng, PhD, Graduate Institute of Biotechnology and Department of Chemical Engineering and Biotechnology, National Taipei University of Technology, Taipei 106, Taiwan, China. wwhlab@gmail.com
Telephone: +886-3-3281022 Fax: +886-3-3285818
Received: September 16, 2010 Revised: November 15, 2010
Accepted: November 22, 2010
Published online: June 28, 2011

Abstract

AIM: To characterize a culture model of rat CCA cells, which were derived from a transplantable TTA-induced CCA and designated as Chang Gung CCA (CGCCA).

METHODS: The CGCCA cells were cultured at *in vitro* passage 12 times on a culture dish in DMEM medium. To measure the doubling time, 10^3 cells were plated in a 96-well plate containing the growth medium. The cells were harvested 4 to 10 d after seeding, and a standard MTT assay was used to measure the growth. The phenotype of CACCA cell and xenograft was determined by immunohistochemical study. We also determine the chromosomal alterations of CGCCA, G-banding and spectral karyotyping studies were performed. The CGCCA cell line was transplanted into the nude mice for examining its tumorigenicity. 2-Deoxy-2-(^{18}F)fluoro-D-glucose (FDG) autoradiography was also performed to evaluate the FDG uptake of the tumor xenograft.

RESULTS: The doubling time for the CGCCA cell line was 32 h. After transplantation into nude mice, FDG autoradiography showed that the tumors formed at the cell transplantation site had a latency period of 4-6 wk with high FDG uptake excluding necrosis tissue. Moreover, immunohistochemical staining revealed prominent cytoplasmic expression of c-erb-B2, CK19, c-Met, COX-Ⅱ, EGFR, MUC4, and a negative expression of K-ras. All data confirmed the phenotypic features of the CGCCA cell line coincide with the xenograft mice tumors, indicating cells containing the tumorigenicity of CCA originated from CCA. In addition, karyotypic banding analysis showed that the diploid (2n) cell status combines with ring and giant rod marker chromosomes in these clones; either both types simultaneously appeared or only one type of marker chromosome in a pair appeared in a cell. The major materials contained in the marker chromosome were primarily identified from chromosome 4.

CONCLUSION: The current CGCCA cell line may be used as a non-K-ras effect CCA model and to obtain information and reveal novel pathways for CCA. Further applications regarding tumor markers or therapeutic targeting of CCA should be addressed accordingly.

© 2011 Baishideng. All rights reserved.

Key words: Cholangiocarcinoma; Rat cell line; Establishment; Characterization; Thioacetamide

Peer reviewer: Giuseppe Garcea, 13 Kinchley Close, Bradgate Heights, Leicester, LE3 9SE, United Kingdom

Yeh CN, Lin KJ, Chen TW, Wu RC, Tsao LC, Chen YT, Weng WH, Chen MF. Characterization of a novel rat cholangiocarcinoma cell culture model-CGCCA. *World J Gastroenterol* 2011; 17(24): 2924-2932 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2924.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2924

INTRODUCTION

Cholangiocarcinoma is a malignant neoplasm derived from bile duct epithelium (i.e. cholangiocytes). It is characterized by a great diversity of symptoms commonly occurring in the late course of the disease, and therefore making treatment puzzling. The biological behavior of the tumor and early intrahepatic and/or extrahepatic spread limit the efficacy of surgical management to perform a curative resection; although liver transplantation may provide an alternative option for CCA treatment, high rates of recurrence still limit liver transplantation for most CCA patients[1] and usually lead to a poor prognosis. Three- to five-year survival rates, even with resection, remain dismal[2-7]; in addition, neither radiation therapy nor chemotherapy significantly improves long-term survival rates. This cancer is related to a wide range of risk factors, such as infestation with liver flukes, primary sclerosing cholangitis, and hepatolithiasis, that cause the incidence rates of CCA to vary greatly among different areas of the world[8]. However, many data have shown that the incidence and mortality rates of CCA have been rising worldwide over the past several decades, particularly the intrahepatic CCA[9-11].

Therefore, our goal is to identify potential possible diagnostic biomarkers as the investigation of the molecular pathophysiology associated with this disease becomes more and more important and necessary. In our previous study, a thioacetamide (TAA)-induced CCA rat model was successfully established, and serves as a powerful pre-clinical platform for therapeutic and chemoprevention strategies for human CCA[12]. Herein, we further developed the rat CCA tumor cells as a cell line designated as Chang Gung CCA (CGCCA), and then transplanted the cells into a xenograft of nude mice to further confirm the characteristics of this cell line. A series of IHC studies, including CK19, c-Met, COX-Ⅱ, and MUC4, were performed to determine the phenotype of the cell line. The genotype was examined by cytogenetic studies, and the 2-Deoxy-2-(^{18}F)fluoro-D-glucose (FDG)-avid character of the CGCCA xenograft of the nude mice was demonstrated by animal PET. All of the evidence proved that the CGCCA cell line rat was derived from the original primary tumor formed by the TAA carcinogen. Our current work supports the view that the systematic cell cultures may provide a relevant CCA model to study the complex mechanisms involved in CCA by revealing the potential pathogenesis of this disease. In addition, we may be able to determine the possible diagnostic markers for the early detection and diagnosis of this disease.

MATERIALS AND METHODS

Animal study

This study was approved by the experimental animal ethics committee at the Chang Gung Memorial Hospital, Taiwan. The investigation conformed to the US National Institute of Health (NIH) guidelines for the care and use of laboratory animals (Publication No. 85-23, revised 1996). Male Sprague-Dawley (SD) rats weighing 319 ± 14 g were used in the experiments. The rats were housed in an animal room with a 12:12 hour light-dark cycle (light from 08:00 AM to 08:00 PM) at an ambient temperature of 22 ± 1℃. Food and water were available *ad libitum*.

Establishment of TAA-treated rats CGCCA cell line

The cell isolation procedure as described by Lai *et al*[13] was applied with minor modifications in this study. In brief, hyperplastic bile ductular epithelial cells were obtained from the liver of the male Sprague-Dawley (SD) rats treated with TAA 300 mg/L daily after 25 wk of exposure. The isolation of the CGCCA cell line was established from the nest of cholangiocarcinoma by employing differential cell harvesting with 0.05% trypsin and 0.53 mmol/L EDTA (Life Technologies), and then combined with subsequent serial propagation suspended in a cell culture medium composed of DMEM with 100 U/mL penicillin and 100 U/mL streptomycin (basal medium) plus 10% fetal bovine serum[13].

Estimating growth kinetics of cells in vitro

The CGCCA cells were cultured at *in vitro* passage 12 times on a culture dish in DMEM medium containing 10% fetal bovine serum. To measure the doubling time, 10^3 cells were plated in a 96-well plate containing the growth medium as described above. The cells were harvested 4 to 10 d after seeding, and a standard MTT assay was used to measure the growth according to the instruction manual (MTT Cell Growth Assay Kit; #CT01; Chemicon). The doubling time of the cell population was estimated based on the slope angle of the linear regression model for the four time points.

Determine phenotype of CACCA cells and xenograft by immunohistochemical study

CGCCA rat cells were grown on a miniature cell culture vessel chamber, which permits cells to be grown, fixed, stained, and analyzed all on the same slide (#154461 Lab-Tek II Chamber Slide System; Nalge Nunc International, USA). A total of 2000 cells per well were grown over night, rinsed with PBS twice, fixed with 4% PFA for 1 min, and perforated with 1% Tween 20 for 1 min. In addition, we further compared the cells with the xenograft mice tumors. The mice tissues were obtained for the immunoreactivities study once the mice tumors reached a

diameter of 1.0 cm. The slides were then stained according to the routine IHC staining as described previously[14]. In brief, the primary antibodies CK19 (MAB-1675; Millipore; Temecula, CA), K-ras (clone sc-30; Santa Cruz Biotechnology Vision Corporation; Fremont, CA), c-erb-B2, COX-Ⅱ, EGFR, MET, and MUC4 were diluted at 1:200 and 1:400, respectively (clone sc-284; Santa Cruz Biotechnologies; Santa Cruz, CA; M-3563; Dako Cytomation; RB-9072-9; Lab Vision Corporation; Fremont, CA; clone sc-161; Santa Cruz Biotechnology Vision Corporation; Fremont, CA; and 35-4900; Zymed S; San Francisco, CA) and incubated overnight at 4℃. The slides were then washed three times with TBST, mounted, and analyzed under microscope by authors blindly before visualization with the DAKO LSAB2 System (Peroxidase; DAKO A/S; No. K0675). Control slides were incubated with the secondary antibody only.

Cytogenetic study of the CGCCA cell line

To determine the chromosomal alterations of CGCCA, G-banding and spectral karyotyping (SKY) studies were performed. The CGCCA cells were grown under the conditions as described above. After the cells were harvested, a metaphase chromosome spread was prepared for G-banding and SKY analysis[15]. At least ten metaphases were analyzed after G-banding (data not shown). For SKY analysis, 22 differentially labeled chromosome-specific painting probes and Cot-1 DNA were denatured and hybridized to the tumor metaphase chromosomes according to the protocol recommended by the manufacturer (Applied Spectral Imaging; Migdal Haemek, Israel) with some modifications as previously described[15]. Image acquisitions were performed using a SD200 Spectracube system (Applied Spectral Imaging) mounted on a Leica DM2500 microscope with a custom-designed optical filter (SKY-1; Chroma Technology; Brattleboro, VT). The clonality criteria and the karyotype description followed the recommendations of the International System for Human Cytogenetic Nomenclature (ISCN 2005)[16].

Heterotransplantation of CGCCA cells in nude mice

Seven male nude mice (BALB/cA Jcl nu/nu) (6 to 8 wk old; 20 to 22 g body weight) were purchased from Clea Japan (Ohita, Japan). The CGCCA cell line was transplanted into the nude mice for examining its tumorigenicity. Each of the 7 mice received a 50 μL subcutaneous inoculation that contained ten million CGCCA cells suspended in the thigh area. Tumor size was measured with a digimatic caliper, and tumor volume was calculated according to the subcutaneous tissue on the flank of each mouse using the formula $V = 1/6\pi abc$ (a, b, and c indicate the diameter in each axis). The xenograft developed after implantation of CGCCA; 4 to 6 wk static later, all animals were sacrificed by CO_2 asphyxia. The xenograft was dissected, and the histopathology of the xenograft was evaluated as described[12]. The xenografted tumors were compared with the original cell line for morphology changes.

FDG autoradiography

FDG positron emission tomography (PET) is an important imaging technique for the evaluation CCA in humans[17]. To evaluate the different levels of fluorodeoxyglucose uptake of the CGCCA cell line xenograft of the nude mice, FDG autoradiography was performed 4-6 wk after tumor implantation. Animals were food-deprived for 8 h, and 37 MBq of FDG was given intravenously. The animals were sacrificed by decapitation after deep anesthesia with isoflurane 45 min after radiotracer injection. The details of the quantitative autoradiography procedures have been described by the authors[18]. In brief, the target tissues, including liver, tumor, and thigh muscle, were quickly removed and placed on dry ice for fixation. The frozen samples were cut to a 10-μm thickness and mounted on glass slides. The slides were exposed to a phosphor image plate (IP) for 2 h and digitized using FLA5100 scanner (Fuji; Japan, Tokyo). The radioactivities of the adjacent tissue sections (assumed to possess the same radiotracer distribution) were measured by γ counter and decay corrected to the time of injection. The correlations between the radioactivities and IP signal intensities were established. All IP signal intensities were converted to radioactivities by using the following calibration curve. Regions of interest (ROI) were manually drawn around the edge of the tumor xenograft activity by visual inspection. The mean activities were recorded from the entire ROI. The percentage injected dose per gram (%ID/g) was calculated as follows: %ID/g = activity in a gram of tissue (Ct)/injected dose × 100%. The slices were stained with hematoxylin-eosin (HE) for histologic examination.

Statistics analysis

All data are presented as mean ± SD. Group comparisons of FDG uptake among tissues were determined using analysis of variance (ANOVA). All statistical analysis was performed using SPSS computer software (Chicago, IL) and a P value of < 0.05 was considered statistically significant.

RESULTS

Estimating growth kinetics of cells in vitro

To establish a TAA-induced rat cell line, 25 wk of orally administered TAA rat liver tissues were harvested for culturing. CGCCA rat cells showed a typical growth curve that included lag, logarithmic, and stationary phases (as shown in Figure 1). The doubling time of the CGCCA cell line was calculated to compare the growth of 10^3 cells. Under the series surveillance, a cell population doubling time of 32 h was determined (6 points at day 4, 5, 6, 7, 8, and 10). When the CGCCA cell line underwent the 26th *in vitro* passage, we harvested cells to perform the cytogenetic or IHC experiments in the current study. The cell line has been maintained in culture for approximately a year so far. Therefore, viable CGCCA cells have been cryopreserved at almost every *in vitro* passage time, from which new cultures may be established.

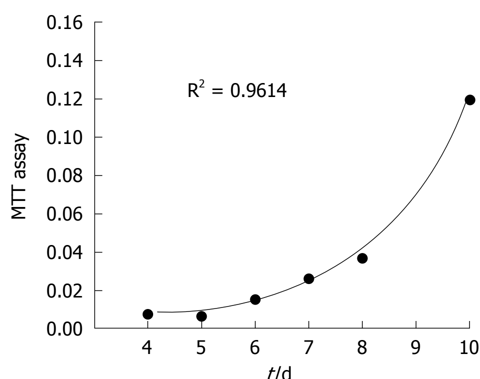


Figure 1 MTT assay. Twenty-five weeks orally administrated TAA rat liver tissues cells have been harvested and successfully cultured. The cells followed a typical growth curve; lag, logarithmic, and stationary phases during culturing were estimated. Under series surveillance, a cell population doubling time of 32 h was determined (6 points at day 4, 5, 6, 7, 8, and 10).

Tumorigenicity study of CCA in transplanted mice

To examine whether the CGCCA cell line contained the tumorigenicity of CCA, the cells were transplanted into the thigh area of seven recipient nude mice and given a 100% incidence of CCA. In addition, their morphological features were essentially identical to those of the parental tumor from which the tumorigenic CCA cells were originally isolated. Tumors formed at the cell transplantation site had a latency period of 4-6 wk, as represented by the photomicrographs shown in Figure 2.

Autoradiography

All seven mice developed a large tumor 4-6 wk after tumor cell implantation. Necropsy and histology confirmed the presence of TAA-induced CCA in 7 mice (Figure 2). Central necrosis could be observed in all large tumors suggesting the fast growth of this malignant tumor. Of note, all tumor cells, excluding necrosis tissue, possessed high FDG uptake. The quantitative uptake value of fluorodeoxyglucose in muscle, liver, and tumor were 0.67 ± 0.17, 2.23 ± 0.85, and 5.00 ± 2.15 %ID/g, respectively (Figure 3A). The tumor to liver and tumor to muscle ratios of FDG uptake were 2.25 ± 0.43 and 7.48 ± 1.78, respectively (Figure 3B). These data are highly consistent with our previous *in vivo* findings, indicating that FDG metabolic activity is significantly higher in the CGCCA xenograft in the nude mice.

The rat CGCCA cell line and heterotransplantation of nude mice tissues were evaluated by immunohistochemistry (IHC) stainings.

To demonstrate the phenotype characteristics and histopathology of CGCCA cells, IHC staining was performed on either the cell line or mouse xenograft tissues. The CGCCA cells revealed prominent cytoplasmic expression of CK19 (biliary cytokeratin 19), as well as c-erb-B2, c-Met, COX-Ⅱ, EGFR, and MUC4 (Figure 4C-H). However, the CGCCA cells revealed a negative expression of K-ras (Figure 4A and B). All of the data from the neoplastic glandular epithelia of the TAA-induced rat CGCCA cell line were highly consistent with previous *in vivo* findings, indicating that these proto-oncogenes are concordantly overexpressed. The tumor tissues from the heterotransplanted nude mice retained the characteristic traits of their *in vitro* cell counterparts. Phenotypically, xenograft highly expressed cytoplasmic immunostaining of CK19, mucin-producing tubular adenocarcinomas closely resembling in their histological and phenotypic features those of the parent tumor; besides, COX-Ⅱ, MET, and MUC4 revealed strong and diffuse cytoplasmic immunostaining as well (Figure 4I-L).

Cytogenetic study of the CGCCA cell line

Spectral karyotyping (SKY) and G-banded analyses were performed to determine the genetic alterations in the CGCCA cell line using the 26th week's TAA-induced rat CGCCA cell line. The cytogenetics study revealed a number of chromosomes ranging between 50 to 56 diploidy (2n) karyotype with complicated genetic abnormalities of marker chromosomes. Obviously, at least two clones were identified in this cell line. In the clones, the loss of whole chromosome 8 and 20 was observed in all analyzed cells; multiple translocated chromosomes formed at the site of chromosome 2q10, such as t(2;3), t(2;5), t(2;3;10); similarly, at the site of chromosome 4q10, commonly formed t(4;13) and t(4;11;4) fusion chromosomes were observed as well. Further, i(6)(q10) was frequently observed in most of the clones. Notably, two major similar contents of ring and giant rod marker chromosomes were involved in these clones; they either simultaneously appeared in a cell or only one type of marker chromosome appeared as a pair to be observed (Figure 5).

DISCUSSION

CCA is the second most common primary hepatic tumor after hepatocellular carcinoma, which is also known as a slowly progressing bile-duct cancer. CCA usually originates in the liver or the intrahepatic bile ducts, and it is characterized by a poor prognosis. Incidence rates account for between 5% and 30% of primary liver cancers and vary geographically[19]. For instance, peripheral CCA represented 3.58% of all primary liver cancers reported by the Japan Liver Cancer Society[1]. According to the report of Bartlett[20], the mortality of intrahepatic CCA has increased 15-fold, and it is currently a more common cause of mortality than hepatocellular carcinoma. New insights into the pathogenesis of this disease, either by using a panel of systematic *in vitro* cell lines for revealing the obscure mechanisms of CCA, or animal models for development of novel therapeutic strategies, are urgently required for clinical-therapeutic purposes.

To our knowledge, there are two published human CCA cell lines available for the purpose of study; however, both have limited availability due to their long-term passage *in vitro*, and they have been shown to be markedly aneuploid. With respect to experimental animal CCA culture models, two published reports have described

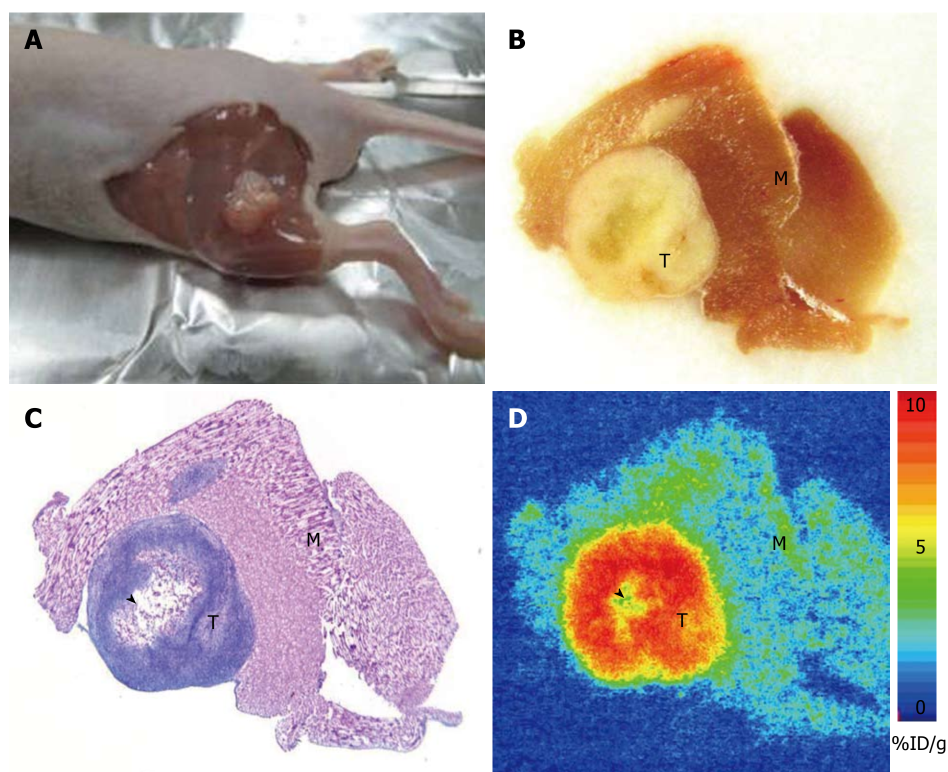


Figure 2 The tumors formed at the cell transplantation site of the CCA xenograft with central necrosis in all large tumors suggesting the fast growth of this malignant tumor at the thigh of the nude mice had a latency period of 4-6 wk and, as represented by (A) gross picture, (B) the photomicrographs of the necropsy, (C) histology, and (D) autoradiography (D). Of note, all tumor cells excluding necrosis tissue possessed high 2-Deoxy-2-(^{18}F)fluoro-D-glucose uptake.

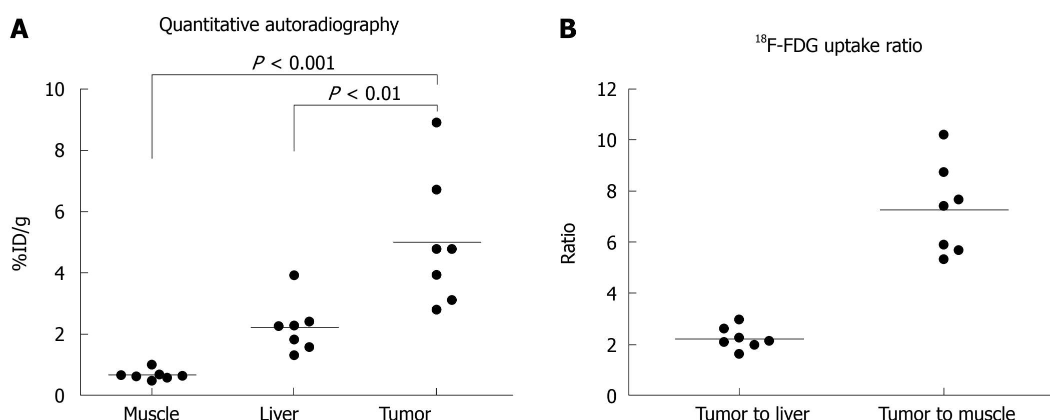


Figure 3 The mean quantitative uptake values of fluorodeoxyglucose in the muscle, liver, and tumor represented by injected dose per gram of tissue (%ID/g) were (A) 0.67 ± 0.17, 2.23 ± 0.85, and 5.00 ± 2.15, respectively. The tumor to liver and tumor to muscle ratios of FDG uptake were (B) 2.25 ± 0.43 and 7.48 ± 1.78, respectively.

the process of establishment; one was developed from liver fluke-associated CCA induced in a hamster model[21]; the other was developed from furan-fed rat CCA culture model[22]. We have been working for several years and have succeeded in establishing a TAA-induced rat intestinal-type CCA model. This model provides a unique and useful study tool for CCA investigation. The important aspects of the cellular and molecular pathogenesis of CCA that are potentially relevant to the human disease have been addressed in previous reports as well[12,23]. In the current study, we further aim to establish a CCA cell line from a TAA rat CCA model and report its identical karyotype as well as immunohistochemical characteristics. This systematic *in vitro* cell line panel will be used as a valuable study tool for revealing the obscure mechanisms of CCA, and searching for the potential biomarkers for CCA tumorigenesis.

Regarding the growth kinetics of the CGCCA cell, CGCCA cells exhibit a 32-h doubling time. This is longer than C611B cells derived from a furan rat model, which exhibited a cell doubling time of approximately 24 h and were aneuploid[13]; but comparable with the hamster liver

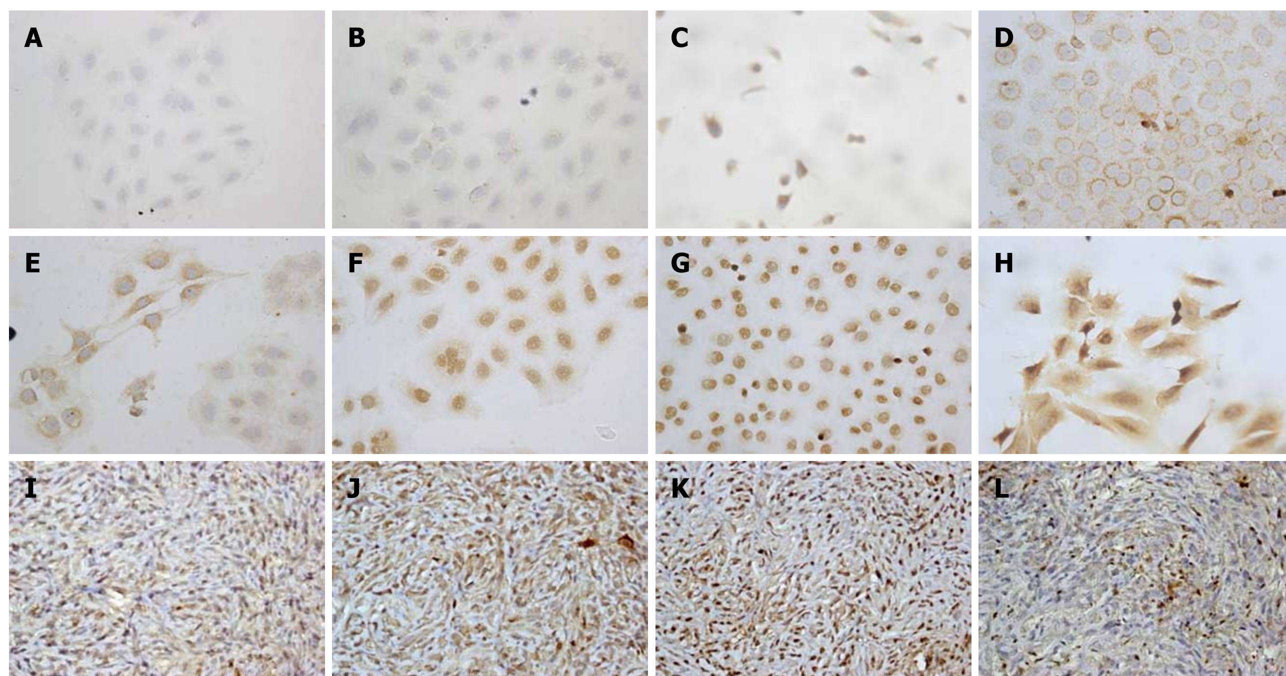


Figure 4 Immunohistochemical staining (× 400) analysis of Chang Gung CCA cells (upper two panels, A-H) and xenograft tissues of CCA mouse models (lower panel, I-L). A: Negative control; B: Negative expression of K-ras; C: EGFR weakly expressed in a cytoplasmic distribution; D-G: Her-Ⅱ, Biliary cytokeratin (CK19), COX-Ⅱ, and Met diffusely expressed in a cytoplasmic distribution; H: MUC-4 strongly and diffusely expressed in a cytoplasmic and membranous distribution; I-L: The results revealed that CK-19, COX-Ⅱ, Met, and MUC4 are diffusely expressed in a cytoplasmic distribution in the rat CCA xenograft.

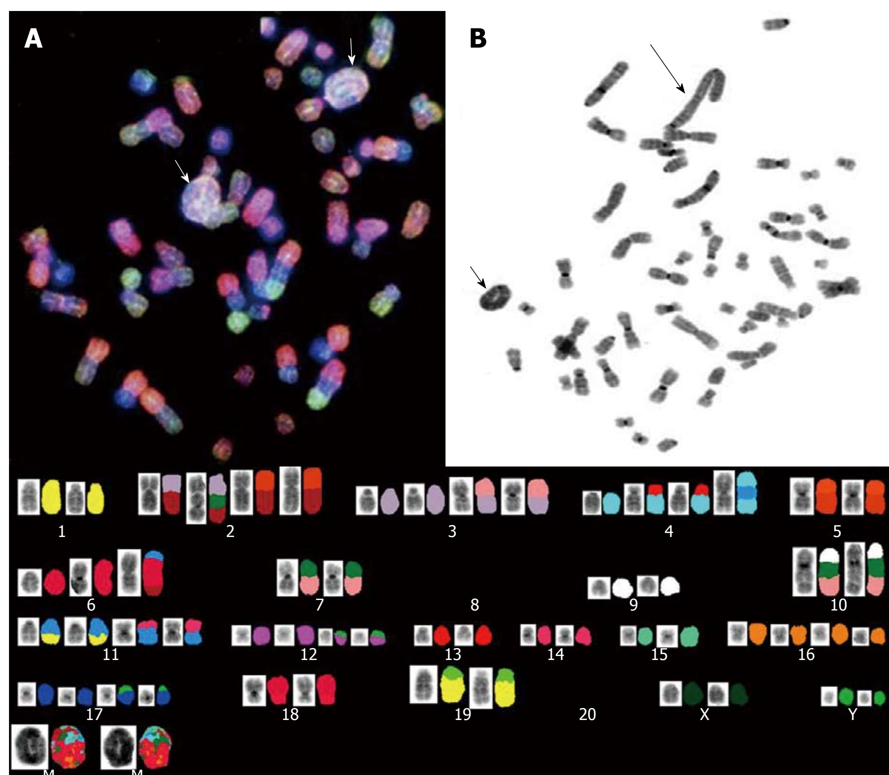


Figure 5 Examples of the karyotype spectral karyotyping analysis revealed by the 26th passage cell culture; the analysis established that, from the 25th week, TAA-induced CCA rat cells presented either (A) a ring chromosome (short arrows) or (B) giant rod chromosome (long arrow); complicated chromosomal alterations could be observed in most of the chromosomes and are illustrated at the bottom.

fluke-associated CCA cells[21]. While skeptics question whether human population doubling times can be slower than animal cell lines, after comparing the population doubling times with some human CCA cell lines cultured

under similar conditions, the human cell lines presented range from 50 to 180 h[24-27].

Regarding phenotype, the strong and diffuse expression of biliary cytokeratin (CK19) confirms the bile ductular ontogeny of the CGCCA cells and xenograft. The molecular alterations involved in CGCCA cells are similar to TAA rat models previously described[12,22,28,29]; the receptor tyrosine kinases c-Met, c-erb-B2 (also known as HER-2/neu), and EGFR and their interact elements, COX-Ⅱ, MUC4, were over-expressed in the current neoplastic cells, either in CGCCA cells or xenograft tissues. It is well known that receptor tyrosine kinases have become important therapeutic targets for anti-tumor molecularly targeted therapies. There is increasing evidence to suggest that, when the HGF/SF interacts with the receptor tyrosine kinases c-Met, the activation could lead to a plethora of biological and biochemical effects in the cell[30] and may play an important role in the development and/or progression of human CCA[31-34]. Other molecular alterations include the overexpression of COX-2[5,35,36] and MUC4, which acts as a ligand for c-erb-B2 and was also confirmed to be overexpressed in the CGCCA cells in our current study. Furthermore, we recently demonstrated that MUC4 could be an independent risk factor of poor prognosis in clinical patients with the mass-forming type of intrahepatic CCA who underwent hepatectomy[37].

Notably, negative K-ras expression was observed in the current CGCCA cells. It is known that very high frequency multiple K-ras gene mutations at codon 12 are commonly detected in CCA (15/15 showed one mutation, and 9/15 showed more than two mutations)[38]. Evidence has further indicated that the mutation status of the K-ras gene affects the response of cetuximab, an epidermal growth factor receptor (EGFR) inhibitor[39]. Therefore, K-ras is considered to be one of the important factors involved in the stepwise progression of neoplastic cells to full malignancy. It is also proven to be related to the higher incidence of bile duct cancers that arise distally in the common bile duct[40,41]. Therefore, the current CGCCA cell line could offer a valuable suitable model that could avoid the influence from K-ras expression.

Nevertheless, evidence of the overexpression of these typical oncoproteins in the CGCCA cell line provides strong resemblances with the human CCA disease, as well as an avenue for future pathogenetic or pharmacologic studies.

In addition, there is evidence that FDG PET imaging may be useful in the diagnosis and management of both hilar and peripheral cholangiocarcinomas in humans[17]. We have previously shown that the FDG uptake pattern in TAA-induced rat CCA was similar to that observed in other human studies[18]. In the present study, our CGCCA xenograft closely mimicked the TAA-induced rat CCA with regard to fluorodeoxyglucose uptake as evaluated by FDG autoradiography. The xenograft had a high tumor to liver and tumor to muscle FDG uptake ratio, which makes *in vivo* tumor detection possible by FDG microPET. The data of the animal PET with regard to the TAA mice models were highly consistent with our previously published *in vivo* findings, indicating that FDG metabolic activity is significantly higher in the CGCCA xenograft in the nude mice[18].

The results of cytogenetic analysis with regard to CGCCA cells revealed complicated chromosomal alterations, and the hyperdiploid karyotype has been characterized in the CGCCA cell line. Basically, the hyperdiploid karyotype is thought to arise from the maintenance of heterozygosity. According to the marker chromosomes observed in the CGCCA cell line, at least three clones could be identified in current cell line, suggesting that the hyperdiploidy does not arise from a near haploid precursor. In addition, although hyperdiploid clones have been identified, all the clones tend to show a pattern of chromosome loss with all copies of chromosomes 8 and 20; further, the gains are more often tetrasomic than trisomic for the chromosomes 2, 3, 4, 11, 12, 16, and 17 (Figure 5). Notably, the marker rings and/or giant rod chromosomes could be observed in every cell as well; the materials contained in the marker chromosomes of the ring or rod shapes varied from cell to cell; however, the major materials mostly came from chromosome 4 as was demonstrated by SKY (Figure 5). To our knowledge, rings are rare in benign tumors, whereas they are common in certain invasive tumors. In addition, the ring chromosomes are even common in certain tumors, especially in subgroups of sarcomas where they may be used as diagnostic indicators for these lesions as described by Gisselsson *et al*[42]. The other additional anomalies, such as translocations and other structural chromosome abnormalities, were present in approximately half of the cells; apparently, the presence of non-random alterations in every clone is most likely the primary change event; in addition, the duplication of chromosome 4 fragments are the most common additional change; deletion of chromosomes 8 and 20, and other random structural abnormalities, such as der(2)t(2;3)(q10;q2), der(2)t(3;10;2), der(2)t(2;5)(q10;q1), der(4)t(4;13)(q10;q?) × 2, der(4)t(4;11;4), are probably a secondary event (Figure 5).

However, although the presence of non-random translocations, such as der(3)t(3;7)(q10;q13), der(3)t(3;7)(q10;q13), der(5)t(5;8)(q10;q1), der(7)t(7;10)(q10;q1), der(11)t(11;1)(q10;?), der(11)t(11;14)(q10;?), der(11)t(11;14)(q10;?), der(17)t(17;Y), der(18)t(18;6)(p10;q1), der(19)t(19;1)(q21;qter), indicates the occurrence of translocation and hyperdiploidy, none of them are known have clinical prognostic implications so far. Therefore, to improve the classification of the disease according to translocation rather than diploidy group in order to assign the correct prognostic implications, further cytogenetic studies to compare the disease stages combined with a series of clinical data for the evaluation of cases are necessary.

Accordingly, our findings suggest that the genes involved in the ring marker chromosomes could play a role in the early stage of tumor development. The correlation

between the conjecture of the cytogenetic changes and tumor progression was also consistent with our previous PET findings. We detected that a 100% initial visual yield of invasive CCA was observed by the 22nd week in rats; however, a 50% yield rate of invasive CCA was observed by the 16th week, and the occurrence of biliary dysplasia and invasive CCA precedes the development of hepatic fibrosis by 4 wk[12].

In conclusion, the current CGCCA cell line was well established and characterized in order to obtain information regarding diagnostically useful tumor markers, which could shed light on a dark area of CCA tumorigenesis for a future understanding of human clinical therapeutics.

COMMENTS

Background

Cholangiocarcinoma is characterized by a great diversity of symptoms commonly occurring in the late course of the disease, and therefore making treatment puzzling. In addition, neither radiation therapy nor chemotherapy significantly improves long-term survival rates. However, many data have shown that the incidence and mortality rates of CCA have been rising worldwide over the past several decades, particularly the intrahepatic CCA. Therefore, the goal is to identify potential possible diagnostic biomarkers as the investigation of the molecular pathophysiology associated with this disease becomes more and more important and necessary. Herein, the authors developed the rat CCA tumor cells as a cell line designated as Chang Gung CCA (CGCCA).

Research frontiers

Positive immunostaining of CK19, c-Met, COX-Ⅱ, and MUC4 determined the phenotype of the cell line. The genotype was examined by cytogenetic studies, and the 2-Deoxy-2-(^{18}F)fluoro-D-glucose-avid character of the CGCCA xenograft of the nude mice was demonstrated by animal PET. All of the evidence proved that the CGCCA cell line rat was derived from the original primary tumor formed by the TAA carcinogen.

Innovations and breakthroughs

The current work supports the view that the systematic cell cultures may provide a relevant CCA model to study the complex mechanisms involved in CCA by revealing the potential pathogenesis of this disease. In addition, the authors may be able to determine the possible diagnostic markers for the early detection and diagnosis of this disease.

Applications

The current CGCCA cell line was well established and characterized in order to obtain information regarding diagnostically useful tumor markers, which could shed light on a dark area of CCA tumorigenesis for a future understanding of human clinical therapeutics.

Peer review

The authors describe the characterization of a new cholangiocarcinoma cell line model and report its successful implantation into nude mice. The paper is well-written, clear and concise.

REFERENCES

1 **Liver Cancer Study Group of Japan**. Classification of primary liver cancer. 1st English ed. Tokyo: Kanehara Shuppan, 1997

2 **Weber SM**, Jarnagin WR, Klimstra D, DeMatteo RP, Fong Y, Blumgart LH. Intrahepatic cholangiocarcinoma: resectability, recurrence pattern, and outcomes. *J Am Coll Surg* 2001; **193**: 384-391

3 **Uenishi T**, Hirohashi K, Kubo S, Yamamoto T, Hamba H, Tanaka H, Kinoshita H. Histologic factors affecting prognosis following hepatectomy for intrahepatic cholangiocarcinoma. *World J Surg* 2001; **25**: 865-869

4 **Jarnagin WR**, Fong Y, DeMatteo RP, Gonen M, Burke EC, Bodniewicz BS J, Youssef BA M, Klimstra D, Blumgart LH. Staging, resectability, and outcome in 225 patients with hilar cholangiocarcinoma. *Ann Surg* 2001; **234**: 507-517; discussion 517-519

5 **de Groen PC**, Gores GJ, LaRusso NF, Gunderson LL, Nagorney DM. Biliary tract cancers. *N Engl J Med* 1999; **341**: 1368-1378

6 **Patel T**. Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. *Hepatology* 2001; **33**: 1353-1357

7 **Shirabe K**, Shimada M, Harimoto N, Sugimachi K, Yamashita Y, Tsujita E, Aishima S. Intrahepatic cholangiocarcinoma: its mode of spreading and therapeutic modalities. *Surgery* 2002; **131**: S159-S164

8 **Shaib Y**, El-Serag HB. The epidemiology of cholangiocarcinoma. *Semin Liver Dis* 2004; **24**: 115-125

9 **Shaib YH**, Davila JA, McGlynn K, El-Serag HB. Rising incidence of intrahepatic cholangiocarcinoma in the United States: a true increase? *J Hepatol* 2004; **40**: 472-477

10 **Patel T**. Worldwide trends in mortality from biliary tract malignancies. *BMC Cancer* 2002; **2**: 10

11 **Chang KY**, Chang JY, Yen Y. Increasing incidence of intrahepatic cholangiocarcinoma and its relationship to chronic viral hepatitis. *J Natl Compr Canc Netw* 2009; **7**: 423-427

12 **Yeh CN**, Maitra A, Lee KF, Jan YY, Chen MF. Thioacetamide-induced intestinal-type cholangiocarcinoma in rat: an animal model recapitulating the multi-stage progression of human cholangiocarcinoma. *Carcinogenesis* 2004; **25**: 631-636

13 **Lai GH**, Sirica AE. Establishment of a novel rat cholangiocarcinoma cell culture model. *Carcinogenesis* 1999; **20**: 2335-2340

14 **Yeh CN**, Pang ST, Chen TW, Wu RC, Weng WH, Chen MF. Expression of ezrin is associated with invasion and dedifferentiation of hepatitis B related hepatocellular carcinoma. *BMC Cancer* 2009; **9**: 233

15 **Weng WH**, Wejde J, Ahlén J, Pang ST, Lui WO, Larsson C. Characterization of large chromosome markers in a malignant fibrous histiocytoma by spectral karyotyping, comparative genomic hybridization (CGH), and array CGH. *Cancer Genet Cytogenet* 2004; **150**: 27-32

16 **Shaffer LG**, Tommerup N. An international system for human cytogenetic nomenclature. Basel: S. Karger, 2005

17 **Fritscher-Ravens A**, Bohuslavizki KH, Broering DC, Jenicke L, Schäfer H, Buchert R, Rogiers X, Clausen M. FDG PET in the diagnosis of hilar cholangiocarcinoma. *Nucl Med Commun* 2001; **22**: 1277-1285

18 **Yeh CN**, Lin KJ, Hsiao IT, Yen TC, Chen TW, Jan YY, Chung YH, Lin CF, Chen MF. Animal PET for thioacetamide-induced rat cholangiocarcinoma: a novel and reliable platform. *Mol Imaging Biol* 2008; **10**: 209-216

19 **Chen MF**. Peripheral cholangiocarcinoma (cholangiocellular carcinoma): clinical features, diagnosis and treatment. *J Gastroenterol Hepatol* 1999; **14**: 1144-1149

20 **Bartlett DL**. Intrahepatic cholangiocarcinoma: a worthy challenge. *Cancer J* 2009; **15**: 255-256

21 **Tengchaisri T**, Prempracha N, Thamavit W, Boonpucknavig S, Sriurairatana S, Sirisinha S. Establishment and characterization of cell lines from liver fluke-associated cholangiocarcinoma induced in a hamster model. *Southeast Asian J Trop Med Public Health* 1995; **26**: 231-239

22 **Sirica AE**. Biliary proliferation and adaptation in furan-induced rat liver injury and carcinogenesis. *Toxicol Pathol* 1996; **24**: 90-99

23 **Jan YY**, Yeh TS, Yeh JN, Yang HR, Chen MF. Expression of epidermal growth factor receptor, apomucins, matrix metalloproteinases, and p53 in rat and human cholangiocarcinoma: appraisal of an animal model of cholangiocarcinoma. *Ann Surg* 2004; **240**: 89-94

24 **Yamaguchi N**, Morioka H, Ohkura H, Hirohashi S, Kawai K. Establishment and characterization of the human cholangiocarcinoma cell line HChol-Y1 in a serum-free, chemically defined medium. *J Natl Cancer Inst* 1985; **75**: 29-35

25 **Miyagiwa M**, Ichida T, Tokiwa T, Sato J, Sasaki H. A new human cholangiocellular carcinoma cell line (HuCC-T1) producing carbohydrate antigen 19/9 in serum-free medium. *In Vitro Cell Dev Biol* 1989; **25**: 503-510

26 **Sirisinha S**, Tengchaisri T, Boonpucknavig S, Prempracha N, Ratanarapee S, Pausawasdi A. Establishment and characterization of a cholangiocarcinoma cell line from a Thai patient with intrahepatic bile duct cancer. *Asian Pac J Allergy Immunol* 1991; **9**: 153-157

27 **Shimizu Y**, Demetris AJ, Gollin SM, Storto PD, Bedford HM, Altarac S, Iwatsuki S, Herberman RB, Whiteside TL. Two new human cholangiocarcinoma cell lines and their cytogenetics and responses to growth factors, hormones, cytokines or immunologic effector cells. *Int J Cancer* 1992; **52**: 252-260

28 **Elmore LW**, Sirica AE. “Intestinal-type” of adenocarcinoma preferentially induced in right/caudate liver lobes of rats treated with furan. *Cancer Res* 1993; **53**: 254-259

29 **Radaeva S**, Ferreira-Gonzalez A, Sirica AE. Overexpression of C-NEU and C-MET during rat liver cholangiocarcinogenesis: A link between biliary intestinal metaplasia and mucin-producing cholangiocarcinoma. *Hepatology* 1999; **29**: 1453-1462

30 **Maulik G**, Shrikhande A, Kijima T, Ma PC, Morrison PT, Salgia R. Role of the hepatocyte growth factor receptor, c-Met, in oncogenesis and potential for therapeutic inhibition. *Cytokine Growth Factor Rev* 2002; **13**: 41-59

31 **Terada T**, Nakanuma Y, Sirica AE. Immunohistochemical demonstration of MET overexpression in human intrahepatic cholangiocarcinoma and in hepatolithiasis. *Hum Pathol* 1998; **29**: 175-180

32 **Endo K**, Yoon BI, Pairojkul C, Demetris AJ, Sirica AE. ERBB-2 overexpression and cyclooxygenase-2 up-regulation in human cholangiocarcinoma and risk conditions. *Hepatology* 2002; **36**: 439-450

33 **Hansel DE**, Rahman A, Hidalgo M, Thuluvath PJ, Lillemoe KD, Shulick R, Ku JL, Park JG, Miyazaki K, Ashfaq R, Wistuba II, Varma R, Hawthorne L, Geradts J, Argani P, Maitra A. Identification of novel cellular targets in biliary tract cancers using global gene expression technology. *Am J Pathol* 2003; **163**: 217-229

34 **Sirica AE**, Lai GH, Zhang Z. Biliary cancer growth factor pathways, cyclo-oxygenase-2 and potential therapeutic strategies. *J Gastroenterol Hepatol* 2001; **16**: 363-372

35 **Muller D**, Zimmerman SI, Schiller F. Drug metabolism in rat liver injured by thioacetamide. *Arch Toxicol* 1982; **5**: 368-371

36 **Dashti HM**, Mathew TC, Jadaon MM, Ashkanani E. Zinc and liver cirrhosis: biochemical and histopathologic assessment. *Nutrition* 1997; **13**: 206-212

37 **Yeh CN**, Pang ST, Wu RC, Chen TW, Jan YY, Chen MF. Prognostic value of MUC4 for mass-forming intrahepatic cholangiocarcinoma after hepatectomy. *Oncol Rep* 2009; **21**: 49-56

38 **Levi S**, Urbano-Ispizua A, Gill R, Thomas DM, Gilbertson J, Foster C, Marshall CJ. Multiple K-ras codon 12 mutations in cholangiocarcinomas demonstrated with a sensitive polymerase chain reaction technique. *Cancer Res* 1991; **51**: 3497-3502

39 **Xu L**, Hausmann M, Dietmaier W, Kellermeier S, Pesch T, Stieber-Gunckel M, Lippert E, Klebl F, Rogler G. Expression of growth factor receptors and targeting of EGFR in cholangiocarcinoma cell lines. *BMC Cancer* 2010; **10**: 302

40 **Ohashi K**, Tstsumi M, Nakajima Y, Nakano H, Konishi Y. Ki-ras point mutations and proliferation activity in biliary tract carcinomas. *Br J Cancer* 1996; **74**: 930-935

41 **Hidaka E**, Yanagisawa A, Seki M, Takano K, Setoguchi T, Kato Y. High frequency of K-ras mutations in biliary duct carcinomas of cases with a long common channel in the papilla of Vater. *Cancer Res* 2000; **60**: 522-524

42 **Gisselsson D**, Höglund M, Mertens F, Johansson B, Dal Cin P, Van den Berghe H, Earnshaw WC, Mitelman F, Mandahl N. The structure and dynamics of ring chromosomes in human neoplastic and non-neoplastic cells. *Hum Genet* 1999; **104**: 315-325

S- Editor Tian L **L- Editor** Rutherford A **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2933

World J Gastroenterol 2011 June 28; 17(24): 2933-2940
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2011 Baishideng. All rights reserved.

ORIGINAL ARTICLE

α-fetoprotein involvement during glucocorticoid-induced precocious maturation in rat colon

Min Chen, Peng Sun, Xiao-Yan Liu, Dan Dong, Jun Du, Luo Gu, Ying-Bin Ge

Min Chen, Lian Yun Gang Higher Vocational Technical College of Traditional Chinese Medicine, Lianyungang 222006, Jiangsu Province, China
Peng Sun, Dan Dong, Jun Du, Luo Gu, Ying-Bin Ge, Department of Physiology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China
Xiao-Yan Liu, The Laboratory Center for Basic Medical Sciences, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China
Author contributions: Chen M and Sun P performed the animal experiment, RT-PCR and immunofluorescent staining and wrote the paper; Liu XY performed part of RT-PCR and immunofluorescent staining; Dong D and Du J carried out Western blotting and statistical analysis; Ge YB contributed to the the paper writing and experimental design; Gu L and Ge YB contributed equally to this work.
Correspondence to: Ying-Bin Ge, MD, PhD, Department of Physiology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China. ybge@njmu.edu.cn
Telephone: +86-25-86862022 **Fax:** +86-25-86862022
Received: July 1, 2010 **Revised:** February 15, 2011
Accepted: February 22, 2011
Published online: June 28, 2011

Abstract

AIM: To investigate the role of α-fetoprotein (AFP), a cancer-associated fetal glycoprotein, in glucocorticoid-induced precocious maturation in rat colon.

METHODS: Colons from suckling Sprague-Dawley rats were used in this study. Corticosterone acetate at a dose of 100 μg/g body weight was given to normal pups on days 7, 9 and 11 after birth to induce hypercorticoidism. Control animals were injected with identical volumes of normal saline. Some rats receiving corticosterone 7 d after birth were also treated with mifepristone (RU38486), a glucocorticoid cytoplasm receptor antagonist to investigate the effects of glucocorticoids (GCs). The morphological changes of the crypt depth and villous height of the villous zone in colon were observed as indices of colon maturation. Expression levels of AFP in colons were detected by reverse transcriptase polymerase chain reaction and Western blotting. To identify the cellular localization of AFP in developing rat colons, double-immunofluorescent staining was performed using antibodies to specific mesenchymal cell marker and AFP.

RESULTS: Corticosterone increased the crypt depth and villous height in the colon of 8- and 10-d-old rats with hypercorticoidism compared with that in the control animals (120% in 8-d-old rats and 118% in 10-d-old rats in villous height, P = 0.021; 145% in 8-d-old rats and 124% in 10-d-old rats in crypt depth, P = 0.017). These increases were accompanied by an increase of AFP expression in both mRNA and protein (2.5-folds in 8-d-old and 2.5-folds in 10-d-old rats higher than in control animals, P = 0.035; 1.8-folds in 8-d-old and 1.3-folds in 10-d-old rats higher than in control animals, P = 0.023). Increased crypt depth and villous height and increased expression of AFP in the colon of rats with hypercorticoidism were blocked by mifepristone. Both had positive staining for AFP or vimentin, and overlapped in mesenchymal cells at each tested colon.

CONCLUSION: GCs promote the development of rat colon. AFP appears to be involved, in part, in mediating the effects of GCs in the developmental colon.

© 2011 Baishideng. All rights reserved.

Key words: Glucocorticoids; α-fetoprotein; Precocious maturation; Colon; Rat

Peer reviewer: Dr. Frank V Schiødt, MD, Clinic of Internal Medicine I, Bispebjerg Hospital, Bispebjerg Bakke 23, DK-2400 Copenhagen, Denmark

Chen M, Sun P, Liu XY, Dong D, Du J, Gu L, Ge YB. α-fetoprotein involvement during glucocorticoid-induced precocious maturation in rat colon. *World J Gastroenterol* 2011; 17(24): 2933-2940 Available from: URL: http://www.wjgnet.

com/1007-9327/full/v17/i24/2933.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2933

INTRODUCTION

Glucocorticoids (GCs) have been routinely used in clinical practice to prevent diseases of prematurity such as respiratory distress syndrome. GCs treatment may reduce the incidence of necrotizing enterocolitis[1-3], in which a typical immaturity of the intestine[4-6] indicated that GCs may enhance intestinal maturation. Although this regulation seems to be mediated by the GCs receptor pathway, the precise regulatory mechanisms have not yet been documented.

The effect of GCs may require interactions with another tissue or cell type, such as mesenchyme[7]. During late embryogenesis, the mouse colon develops from a pseudostratified, undifferentiated endoderm to a single-layered columnar epithelium accompanying with mesenchymal maturation. Mammalian α-fetoprotein (AFP) is a single-chain glycoprotein with a molecular mass ranging from 66 to 72 kDa and 3%-5% carbohydrate (glycan) content. Recent reports showed that the AFP was expressed and produced in mesenchymal cells and was considered as an important growth factor with a specific function in gastrointestinal development, including pancreas and colon[8-10]. In contrast, AFP enhancer segment contains a sequence resembling the steroid hormone response element and the enhancer activity was mediated by dexamethasone in a dose-dependent manner[11]. Thus, AFP seems to play a role in GCs-induced precocious gastrointestinal maturation.

Numerous investigators have reported the ability of GCs to stimulate intestinal maturation in postnatal rodents[12-14]. However, little is known about the involvement of GCs in rat colon during development.

MATERIALS AND METHODS

Chemicals

Corticosterone acetate (CA), gel mount aqueous mounting medium (G0918), and mifepristone were purchased from Sigma Chemical (St. Louis, USA). TRIZOL reagent was purchased from Invitrogen Life Technologies (Burlington, Ontario, Canada). Takara RNA polymerase chain reaction (PCR) 3.0 Kit was from Takara (Dalian, China). Rabbit anti-goat IgG conjugates and goat polyclonal anti-AFP were from Santa Cruz Biotech (USA). Monoclonal antibody of anti-vimentin was from Chemicon International (Temecula, CA, USA). Protease inhibitor cocktail was from Roche (Mannheim, Germany). Nitrocellulose membranes were from Bio-Rad (Hercules, CA, USA), BCA Protein Assay Kit and enhanced chemiluminescence reagents were from Pierce (Rockford, IL, USA) and CBL202 from Chemicon International, Inc. Temecula (CA, USA).

Animals

Pregnant female Sprague-Dawley rats in late gestation were purchased from Nanjing Medical University Animal Centre. After arrival, cages were checked twice daily for pups. The day of birth was designated as day 0, and experiments were conducted at various ages thereafter. Litter size was restricted to 12 pups per dam that was performed at day 2 postpartum. All animals were kept in a temperature-controlled room (22 ± 1℃) and maintained on a 12-h light-dark cycle. To prevent the stress of removing one animal from a cage on sequential days, all the animals in a cage were killed on a single day. Young rats were never separated from their mothers except during the period of fasting. During this period, the pups were placed in cages artificially warmed by electric light bulbs. Before studies, all rats were fasted for various times. To prevent mortality from prolonged fasting, the 8- and 10-d-old rats were fasted for 18 h, and 14-d-old rats were fasted for 24 h. Experiments were designed using littermate controls disregarding the sex of the pups. All animals were killed between 8 and 10 am[15]. CA was given at a dose of 100 μg/g body weight by intraperitoneal injection to normal pups on days 7, 9, and 11 to induce hypercorticoidism. Control animals were given identical volumes of normal saline[16]. To observe the effect of GCs on suckling rats, another experiment was designed as follows: 100 μg/g body weight of CA was given once to pairs of 7-d-old pups (HC group) by intraperitoneal injection. Control pups (C group) were injected with identical volumes of normal saline at the same day. Pups were treated with 50 μg/g body weight mifepristone (17β-hydroxy-11β-4-dimethyl-aminophenyl-17α-propynylestra-4,9-diene-3-one, RU38486), a glucocorticoid cytoplasm receptor antagonist alone (M group), or 100 μg/g hydrocortisone plus 50 μg/g mifepristone (H+M group)[17].

Colons from control and hypercorticoidism rats at 8, 10 and 14 d of age were used in this study and five rats were used in each age stage. Samples were fixed in 4% paraformaldehyde overnight at 4℃ followed by a standard protocol of dehydration and paraffin embedding. Five-micrometer sections were prepared for morphological and fluorescence immunohistochemical studies. Total RNA and lysate were extracted from tissues at each time point for the reverse transcriptase PCR (RT-PCR) and Western blotting analysis. The study protocol was approved by the Nanjing Medical University Animal Care and Use Committee.

Morphology

Routine hematoxylin and eosin-stained sections for light microscopic (LM) evaluation were used to study the hormonal effects in the morphological development of colon. Slides were viewed under an Olympus BX51 microscope (Olympus Optical, Tokyo, Japan). Morphologic measurement made by the same technician was blinded to the different treatment groups. Under LM, the crypt depth and villous height of the villous zone were mea-

sured using an image analysis system (NYD100). The group means were obtained based on 10 villi and 20 crypts per slide, with a minimum of five animals in each group.

Serial levels of AFP

Blood samples of rats were centrifuged at 2000 × g for 20 min, and sera were stored at -20℃ until analyzed. AFP levels in the rat serum were measured by the routine standard radioactive method used in the Nanjing Clinical Nuclear Medicine Center (Nanjing, China).

RNA expression of AFP

Total RNA was isolated from tissues by Trizol according to the protocol supplied by the manufacturers. cDNA was synthesized using Takara RNA PCR 3.0 Kit in a total volume of 10 μL, containing 0.5 μL avian myeloblastosis virus RT, 0.5 μL random 9 primer, 2 μL 25 mmol/L $MgCL_2$, 1 μL 10 × RT buffer, 1 μL dNTP mixture (each 10 mmol/L), 0.25 μL RNase inhibitor, 1 μL RNA, and 3.75 μL dH_2O. Conditions for RT were: 30℃ for 10 min, 42℃ for 25 min, 99℃ for 5 min, and 5℃ for 5 min. PCR was performed in 50 μL reactions containing 2.5 ng cDNA, 1 μL each primer pair, and 25 μL Premix *Taq* in the Takara RNA PCR kit. PCR was carried out in a T-gradient Biometra PCR thermal cycler (Montreal Biotech Inc., Kirkland, Quebec, Canada) to determine the annealing temperature for each paired primers. The following AFP primer pairs were used: 5'-GCTGAACCCAGAGTACTGCAC-3' (forward), and 5'-GACACGTCGTAGATGAACGTG-3' (reverse). Amplification reactions were carried out for 30 cycles at 94℃ for 30 s, 58.4℃ for 30 s, and at 72℃ for 1 min.

The amplified products were 443 bp and analyzed on 1% agarose gels and visualized by ethidium bromide staining. Omitting RT, cDNA or DNA polymerase were adopted in the controls, and showed no reaction bands. The data were normalized by actin.

Protein expression of AFP

The tissues were homogenized in a sample buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride and protease inhibitor cocktail. An equal amount of protein samples were separated by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After transferred to nitrocellulose membranes and blocked with 5% fat-free milk in Tris-buffered saline plus 0.05% Tween 20 overnight at 4℃, polyclonal antibody for AFP and the corresponding secondary antibody were applied. Blots were visualized with enhanced chemiluminescence reagents and exposed to X-Omat BT film. Signal intensity was quantified using a Bio-Rad image analysis system and the results were normalized to band intensities at e18.5. The β-actin was used as an internal control and the primary antibody was omitted for negative controls.

Regional and cellular localization of AFP

Double-immunofluorescent staining of AFP and vimentin, a specific marker of mesenchymal cell, were used to determine the regional and cellular localization of AFP in rat colons. Staining was performed according to the standard procedures. Briefly, the sections were deparaffinized in xylene, cleared with graded ethanol in phosphate buffered saline (PBS), and then placed in 10 mmol/L citrate buffer (pH 6.0) for 15 min at 100℃ for antigen retrieval. The sections were applied to goat anti-AFP polyclonal antibody overnight at 4℃ and then linked with FITC-labeled rabbit anti goat-IgG. After washing by Tris buffered saline (TBS), mouse anti-vimentin monoclonal antibody and rhodamine-labeled anti-mouse IgG were applied. Sections were placed in Gel Mount aqueous mounting medium with a cover glass and were examined under an Olympus BX51 microscope. Controls were treated by omitting the primary or secondary antibodies. No staining was observed under the negative control conditions. Images were taken at a magnification of × 200. An image analysis system (NYD100) was used for quantitative analysis of cell density (cell number/view field) of the AFP-positive cells in the rat colon. Four sections from four rats in each group were used. AFP-positive cells were counted in five randomly selected view fields per section at a magnification of × 400. At least 20 fields in each group were analyzed.

Statistical analysis

All experiments were done in triplicate. The experimental data was analyzed using PDQuest 7.0 software (Bio-Rad Laboratories, Hercules, CA, USA) and one-way analysis of variance and paired t test were used. Data were presented as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Morphology

Injecting corticosterone into the suckling animals for 1, 2, and 3 d showed no effect on the animal's intestinal and body weights (data not shown). The crypt depth and villous height after corticosterone treatment were higher in the colon of 8- and 10-d-old rats with hypercorticoidism than in the control animals and did not influence those in the 14-d-old rats with hypercorticoidism (Table 1).

Expression of AFP

The serum AFP levels in the rats were near 18 μmol/L in all measured rats and had no difference between hypercorticoidism and control animals (data not shown). In this study, we analyzed the effect of corticosterone on the level of AFP mRNA by RT-PCR. The AFP mRNA concentration increased significantly after corticosterone treatment, by 2.5-folds in the 8-d-old and 2.5-folds in the 10-d-old rats compared with those in the control animals (Figure 1). The transcriptional regulation of AFP expres-

Table 1 Morphological changes in suckling rat colon treated with corticosterone acetate

	Villous height (μm)			Crypt depth (μm)		
	8 d	10 d	14 d	8 d	10 d	14 d
Controls	124.6 ± 41.4	165.9 ± 34.4	254.3 ± 57.7	26.9 ± 10.3	40.4 ± 14.7	53.0 ± 23.5
CA	151.1 ± 35.1[a]	196.9 ± 29.6[a]	248.5 ± 42.3	39.1 ± 5.7[a]	50.3 ± 13.6[a]	60.5 ± 15.6

[a]$P < 0.05$ *vs* control. CA: Corticosterone acetate.

Table 2 Changes in number of α-fetoprotein-positive cells in suckling rat colon treated with corticosterone acetate (numbers/view field, mean ± SD, *n* = 20)

	8 d	10 d	14 d
Controls	21.6 ± 4.7	14.1 ± 4.3	12.3 ± 5.2
CA	38.2 ± 5.2[a]	23.8 ± 2.5[a]	11.5 ± 2.3

[a]$P < 0.05$ *vs* control. CA: Corticosterone acetate.

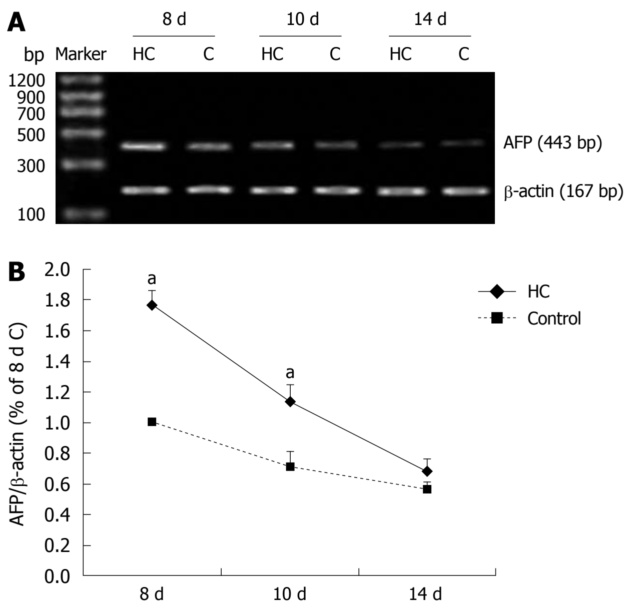


Figure 1 Expression of α-fetoprotein mRNA by reverse transcriptase polymerase chain reaction analysis in colons of suckling rats treated with or without hydrocortisone. Molecular weight markers are indicated on the left. A: The α-fetoprotein (AFP) mRNA levels in colons of hydrocortisone-treated animals were higher than in controls of 8- and 10-d-old rats; B: Results are indicated in percentage above the β-actin value and are representative of three independent experiments. [a]$P < 0.05$ *vs* controls; C: Pups received saline; HC: Pups received hydrocortisone.

sion of corticosterone was confirmed by the Western blotting analysis (Figure 2).

Regional and cellular localization of AFP

The AFP positive cells were scattered on the epithelium in either corticosterone treated or untreated rats. The cell density of AFP-positive cells was higher in 8- and 10-d-old corticosterone-treated rats than in control animals (Table 2). Double-immunofluorescent staining for the vimentin and AFP showed that there was complete overlap between the AFP-positive cells and the antibody staining for vimentin in all the tested animals (Figure 3).

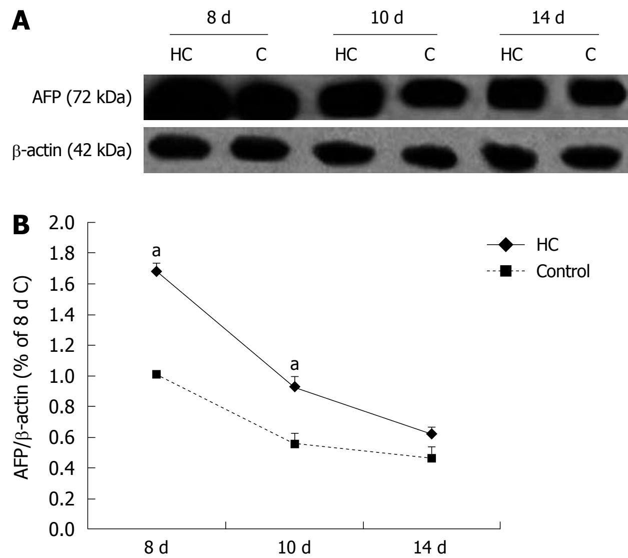


Figure 2 Expression of α-fetoprotein protein by Western blotting analysis in colons of suckling rats treated with or without hydrocortisone as indicated in lanes, respectively. Western blotting analysis using α-fetoprotein (AFP) (C-19), revealed a 72 kDa isoform. A: AFP levels in colons of hydrocortisone-treated animals were higher than in controls of 8- and 10-d-old rats; B: Results are indicated in percentage above the β-actin value and are representative of three independent experiments. [a]$P < 0.05$ *vs* controls; C: Pups received saline; HC: Pups received hydrocortisone.

Effect of mifepristone

The effect of increased crypt depth and villous height in the colon of 8-d-old rats with hypercorticoidism was blocked by mifepristone (Figure 4). The increased expression of AFP was also inhibited in the rats treated with mifepristone (Figure 5).

DISCUSSION

Although rodent colon may differ from human colon in the breadth of its responses to GCs, it is nevertheless a useful model to address issues related to morphological maturation, since the development of gastrointestinal functions is similar in all mammals. The aim of this study was to assess the role of exogenous GCs in the maturation of the colon. Accordingly, we used the rats in the first postnatal week whose circulating concentrations of natural GCs were very low (< 0.5 μg/mL)[18].

Exogenous administration of GCs causes precocious maturation of the intestine. In this study, GCs did result in an increase in villous height and crypt depth of the colon (Table 1) in 8- and 10-d-old rats compared with the age-matched controls. This effect was completely blocked

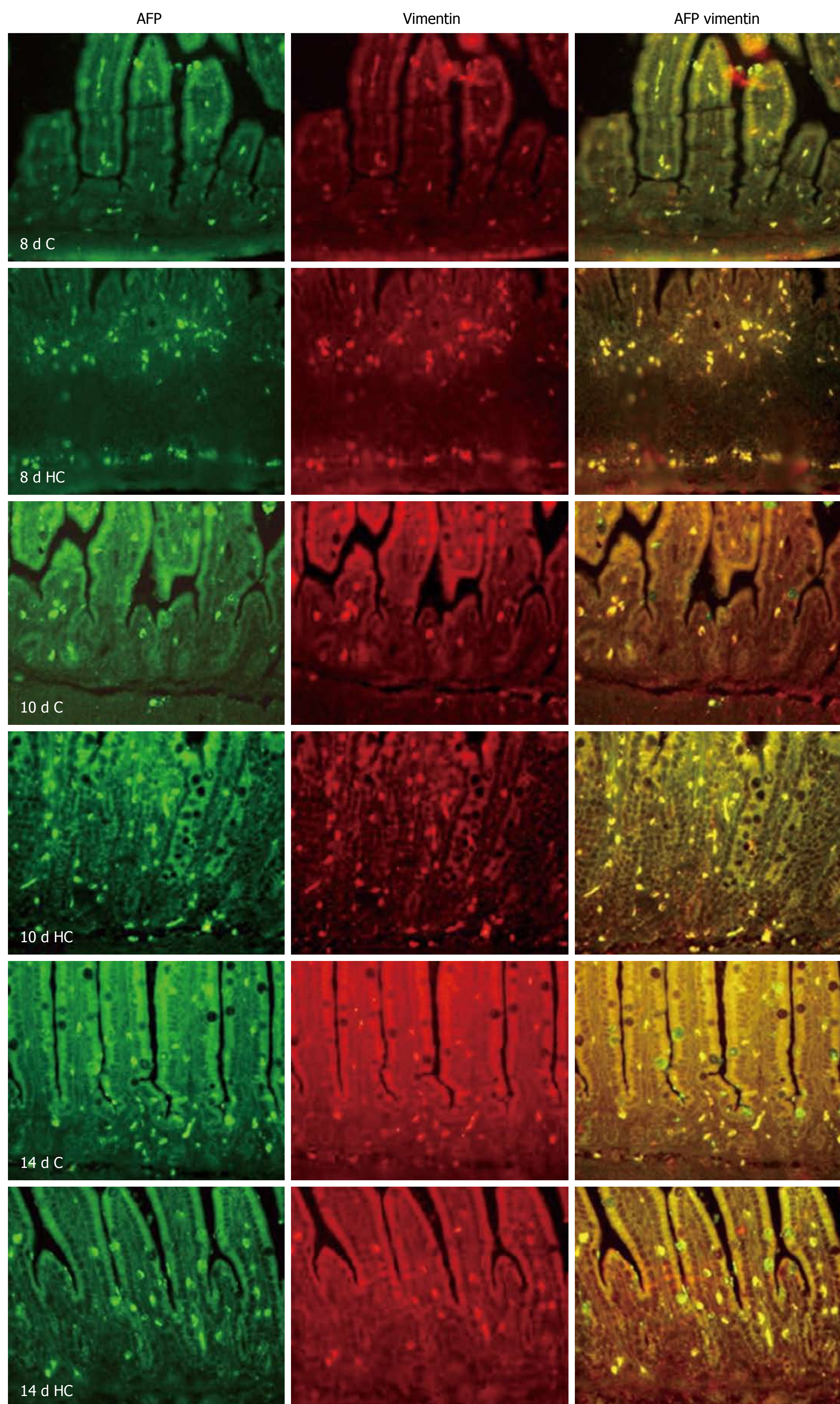


Figure 3 Immunofluorescence localization of α-fetoprotein and vimentin in the suckling rats treated with or without hydrocortisone. Labeling by the α-fetoprotein (AFP) antibody was detected with a fluorescein isothiocyanate (green) labeled secondary antibody. Labeling of vimentin was detected with a rhodamine-(red)-labeled secondary antibody on the same section. The overlap of AFP (green) and vimentin (red) labeling displayed orange color. Double-staining revealed complete colocalization of AFP and vimentin in the same cells of rat colons treated with or without hydrocortisone. All of the primary magnifications are × 200.

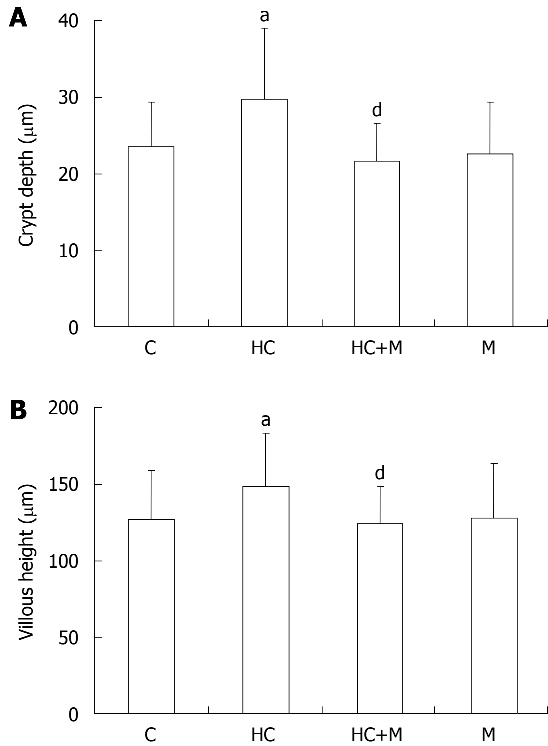


Figure 4 Morphological changes in colons of suckling rats treated with or without hydrocortisone, hydrocortisone + mifepristone and mifepristone alone. Villous height (A) and crypt depth (B) were determined. Each data point (± SD; bars) is the mean of four independent trials. C: Pups received saline; HC: Pups received hydrocortisone; HC+M: Pups treated with both hydrocortisone and mifepristone; M: Pups treated with mifepristone only. $^aP < 0.05$ *vs* control animals; $^dP < 0.01$ *vs* hydrocortisone-treated animals.

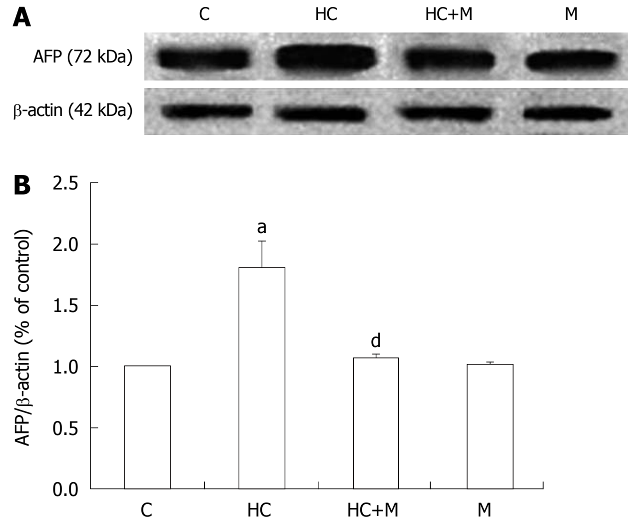


Figure 5 α-fetoprotein expressions in 8-d-old rat colons treated with or without hydrocortisone, hydrocortisone + mifepristone and mifepristone alone. α-fetoprotein (AFP) expression was determined by the Western blotting using AFP polyclonal antibody (A), and results indicated are in percentage above the β-actin value and are representative of three independent experiments (B). Each data point (± SD; bars) is the mean of four independent trials. C: Pups received saline; HC: Pups received hydrocortisone; HC+M: Pups treated with both hydrocortisone and mifepristone; M: Pups treated with mifepristone only. $^aP < 0.05$ *vs* control animals; $^dP < 0.01$ *vs* hydrocortisone-treated animals.

by the mifepristone, a glucocorticoid cytoplasm receptor antagonist (Figure 4). It is possible that GCs had a precocious effect on the development of rat colon in the first postnatal week, and in the rat small intestine as well[19,20].

In contrast, the GCs had no effect on the colon in 14-d-old rats. It has been suggested that immature gastrointestinal tract seems to have more sensitive responsiveness to the regulation of GCs[21]. It is possible that prepartum cellular differentiation progresses until day 10 in rats after birth[22]. On the other hand, cytoplasm receptors of GCs are activated after the binding of GCs that allows their translocation to the nucleus. Of note is the pattern of ontogeny changes in the concentration of GCs receptors in the small intestine as cytoplasm GCs receptors are present in the intestine at all ages, but at higher concentrations during the two postnatal weeks than in older rats[23]. Moreover, GCs receptors themselves have been shown to downregulate by GCs through GCs enhanced expression of a factor (repressor), which binds the regulatory sequence in the GCs receptor gene promoter[24]. This might serve as a negative feedback control in the GCs response systems under developmental conditions. In 14-d-old rats, the plasma corticosterone of GCs was raised to 5 μg/mL[18], and a loss of responsiveness to GCs for colon development might occur in these rats. No effect of exogenous GCs administration could be also explained by a fall in GCs receptors in 14-d-old rats with a natural rise in corticosterone level, although this was not tested in this study.

AFP is known to be associated with the successful completion of term pregnancies in mammals and even minute amounts of AFP may still be necessary during human pregnancy[25]. The capability of both up and down modulation of growth and differentiation as a dose-dependent function of AFP has been demonstrated in a multitude of cell types, including placental, ovarian, uterine, and lymphoid, epidermal, endothelial, testicular, breast, and liver[26-30]. In our previous studies, we indicated that AFP was expressed and produced in mesenchymal cells, which might act as a potent paracrine regulator of colonic cell proliferation and organ maturation[10]. In this study, exogenous administration of GCs demonstrated an increased expression of AFP, this effect was also inhibited by mifepristone (Figures 2 and 3). Double-immunofluorescent staining for the vimentin, a mesenchymal cell marker, and AFP showed that the AFP was localized in the mesenchymal cells both in GCs-treated and control colons, similar to our previous study[10].

In present study, the AFP levels in the rat serum were near 18 μmol/L in all measured rats and had no difference between hypercorticoidism and control animals (data not shown). High cortisone concentration in serum prompts the acceleration of colonic ontogenesis, and is not accompanied by an increased level of serial AFP. In the current study, we did observe an increased expression of AFP in both RNA and protein levels in the GCs-treated premature colon of rats. Therefore, mesenchymal cell-derived AFP acted as a potent paracrine regulator in rat developmental colon.

The effect of GCs may require interactions with another tissue or cell type, such as mesenchyme[7]. The epithelial-mesenchymal interactions play an essential role in the control of gastrointestinal epithelial growth and differentiation not only in fetal stages, but also in adults[31,32]. There is no report that GCs could regulate the growth colonic mucosa directly. Our *in vitro* study also showed a negative result for GCs stimulating the proliferation of colonic epidermis (data not shown). The exogenous GCs were blocked, and the effect of accelerating maturation and the increased expression of AFP were also inhibited. Therefore, mesenchymal cell-derived AFP appears to be responsible, in part, in mediating the effects of GCs on developmental colon. Our present study may help us discern whether the epithelial-mesenchymal interactions and the effect of GCs enhance intestinal maturation in the gastrointestinal tract.

The involvement of GCs in AFP expression in the gut still remains an open question. Studies are now in progress in our laboratory using animal and tissue culture models to test it.

In summary, our present study for the first time demonstrated that GCs promote the development of rat colon. AFP appears to be responsible, in part, in mediating the effects of GCs on developmental colon. The exact function of AFP in rat colon development remains to be determined.

COMMENTS

Background

Glucocorticoids (GCs) are considered to play an important role in the maturation of the gastrointestinal (GI) tract. However, the mechanism of GCs on GI system development has not been fully elucidated.

Research frontiers

Although rodent colon may differ from human colon in the breadth of its responses to GCs, it is nevertheless a useful model to address issues related to morphological maturation, since the development of gastrointestinal functions is similar in all mammals. This study assessed the role of exogenous GCs in the maturation of the colon in rats.

Innovations and breakthroughs

This study for the first time demonstrated that GCs promote the development of rat colon. α-fetoprotein (AFP) appears to be responsible, in part, in mediating the effects of GCs on developmental colon. The exact function of AFP in rat colon development remains to be determined.

Applications

This animal model is a useful tool for the study of mechanism of gastrointestinal mucosal proliferation and differentiation *in vivo*.

Terminology

Corticosterone acetate is a chemical compound of GCs. Mammalian AFP is a single-chain glycoprotein with a molecular mass ranging from 66 to 72 kDa and 3%-5% carbohydrate (glycan) content.

Peer review

This is an excellent study.

REFERENCES

1 **Bauer CR**, Morrison JC, Poole WK, Korones SB, Boehm JJ, Rigatto H, Zachman RD. A decreased incidence of necrotizing enterocolitis after prenatal glucocorticoid therapy. *Pediatrics* 1984; **73**: 682-688

2 **Crowley P**, Chalmers I, Keirse MJ. The effects of corticosteroid administration before preterm delivery: an overview of the evidence from controlled trials. *Br J Obstet Gynaecol* 1990; **97**: 11-25

3 **Tapia JL**, Ramírez R, Cifuentes J, Fabres J, Hübner ME, Bancalari A, Mercado ME, Standen J, Escobar M. The effect of early dexamethasone administration on bronchopulmonary dysplasia in preterm infants with respiratory distress syndrome. *J Pediatr* 1998; **132**: 48-52

4 **Crissinger KD**. Animal models of necrotizing enterocolitis. *J Pediatr Gastroenterol Nutr* 1995; **20**: 17-22

5 **Kliegman RM**. Models of the pathogenesis of necrotizing enterocolitis. *J Pediatr* 1990; **117**: S2-S5

6 **Israel EJ**. Neonatal necrotizing enterocolitis, a disease of the immature intestinal mucosal barrier. *Acta Paediatr Suppl* 1994; **396**: 27-32

7 **Tsukada S**, Ichinose M, Yahagi N, Matsubara Y, Yonezawa S, Shiokawa K, Furihata C, Miki K, Fukamachi H. Induction of precocious pepsinogen synthesis by glucocorticoids in fetal rat gastric epithelium in organ culture: importance of mesenchyme for epithelial differentiation. *Differentiation* 1998; **62**: 239-247

8 **Angeletti RH**. Chromogranins and neuroendocrine secretion. *Lab Invest* 1986; **55**: 387-390

9 **Liu L**, Guo J, Yuan L, Cheng M, Cao L, Shi H, Tong H, Wang N, De W. Alpha-fetoprotein is dynamically expressed in rat pancreas during development. *Dev Growth Differ* 2007; **49**: 669-681

10 **Liu XY**, Dong D, Sun P, Du J, Gu L, Ge YB. Expression and location of alpha-fetoprotein during rat colon development. *World J Gastroenterol* 2009; **15**: 1738-1743

11 **Houart C**, Szpirer J, Szpirer C. The alpha-foetoprotein proximal enhancer: localization, cell specificity and modulation by dexamethasone. *Nucleic Acids Res* 1990; **18**: 6277-6282

12 **Zhou YJ**, Gao J, Yang HM, Zhu JX, Chen TX, He ZJ. Morphology and ontogeny of dendritic cells in rats at different development periods. *World J Gastroenterol* 2009; **15**: 1246-1253

13 **Galand G**. Brush border membrane sucrase-isomaltase, maltase-glucoamylase and trehalase in mammals. Comparative development, effects of glucocorticoids, molecular mechanisms, and phylogenetic implications. *Comp Biochem Physiol B* 1989; **94**: 1-11

14 **Yeh KY**, Yeh M, Holt PR. Thyroxine and cortisone cooperate to modulate postnatal intestinal enzyme differentiation in the rat. *Am J Physiol* 1991; **260**: G371-G378

15 **Tseng CC**, Johnson LR. Does corticosterone affect gastric mucosal cell growth during development? *Am J Physiol* 1986; **250**: G633-G638

16 **Tseng CC**, Schmidt KL, Johnson LR. Hormonal effects on development of the secretory apparatus of chief cells. *Am J Physiol* 1987; **253**: G274-G283

17 **Fiancette JF**, Balado E, Piazza PV, Deroche-Gamonet V. Mifepristone and spironolactone differently alter cocaine intravenous self-administration and cocaine-induced locomotion in C57BL/6J mice. *Addict Biol* 2010; **15**: 81-87

18 **Henning SJ**. Plasma concentrations of total and free corticosterone during development in the rat. *Am J Physiol* 1978; **235**: E451-E456

19 **Biol-N'garagba MC**, Niepceron E, Mathian B, Louisot P. Glucocorticoid-induced maturation of glycoprotein galactosylation and fucosylation processes in the rat small intestine. *J Steroid Biochem Mol Biol* 2003; **84**: 411-422

20 **Quaroni A**, Tian JQ, Göke M, Podolsky DK. Glucocorticoids have pleiotropic effects on small intestinal crypt cells. *Am J Physiol* 1999; **277**: G1027-G1040

21 **Solomon NS**, Gartner H, Oesterreicher TJ, Henning SJ. Development of glucocorticoid-responsiveness in mouse intestine. *Pediatr Res* 2001; **49**: 782-788

22 **Helander HF**. Morphological studies on the development of the rat colonic mucosa. *Acta Anat (Basel)* 1973; **85**: 155-176

23 **Henning SJ**, Ballard PL, Kretchmer N. A study of the cyto-

plasmic receptors for glucocorticoids in intestine of pre- and postweanling rats. *J Biol Chem* 1975; **250**: 2073-2079

24 **LeClerc S**, Palaniswami R, Xie BX, Govindan MV. Molecular cloning and characterization of a factor that binds the human glucocorticoid receptor gene and represses its expression. *J Biol Chem* 1991; **266**: 17333-17340

25 **Sher C**, Shohat M. Congenital deficiency of AFP and Down syndrome screening. *Prenat Diagn* 1997; **17**: 884-885

26 **Keel BA**, Eddy KB, Cho S, May JV. Human alpha-fetoprotein purified from amniotic fluid enhances growth factor-mediated cell proliferation in vitro. *Mol Reprod Dev* 1991; **30**: 112-118

27 **Wang XW**, Xie H. Alpha-fetoprotein enhances the proliferation of human hepatoma cells in vitro. *Life Sci* 1999; **64**: 17-23

28 **Cingolani N**, Shaco-Levy R, Farruggio A, Klimstra DS, Rosai J. Alpha-fetoprotein production by pancreatic tumors exhibiting acinar cell differentiation: study of five cases, one arising in a mediastinal teratoma. *Hum Pathol* 2000; **31**: 938-944

29 **Edlund H**. Developmental biology of the pancreas. *Diabetes* 2001; **50 Suppl 1**: S5-S9

30 **De Mees C**, Laes JF, Bakker J, Smitz J, Hennuy B, Van Vooren P, Gabant P, Szpirer J, Szpirer C. Alpha-fetoprotein controls female fertility and prenatal development of the gonadotropin-releasing hormone pathway through an antiestrogenic action. *Mol Cell Biol* 2006; **26**: 2012-2018

31 **Kedinger M**, Simon-Assmann PM, Lacroix B, Marxer A, Hauri HP, Haffen K. Fetal gut mesenchyme induces differentiation of cultured intestinal endodermal and crypt cells. *Dev Biol* 1986; **113**: 474-483

32 **Sanderson IR**, Ezzell RM, Kedinger M, Erlanger M, Xu ZX, Pringault E, Leon-Robine S, Louvard D, Walker WA. Human fetal enterocytes in vitro: modulation of the phenotype by extracellular matrix. *Proc Natl Acad Sci USA* 1996; **93**: 7717-7722

S- Editor Sun H **L- Editor** Ma JY **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2941

World J Gastroenterol 2011 June 28; 17(24): 2941-2944
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2011 Baishideng. All rights reserved.

BRIEF ARTICLE

Is hyperhomocysteinemia relevant in patients with celiac disease?

Giovanni Casella, Gabrio Bassotti, Vincenzo Villanacci, Camillo Di Bella, Fabio Pagni, Gian Luigi Corti, Giuseppe Sabatino, Mara Piatti, Vittorio Baldini

Giovanni Casella, Vittorio Baldini, Division of Internal Medicine, Desio General Hospital, 20033 Desio, Italy
Gabrio Bassotti, Giuseppe Sabatino, Gastroenterology and Hepatology Section, Department of Clinical and Experimental Medicine, Ospedale Santa Maria della Misericordia, Piazzale Menghini, 1, 06156 San Sisto (Perugia), Italy
Vincenzo Villanacci, 2nd Pathology Section, Spedali Civili, 25100 Brescia, Italy
Camillo Di Bella, Fabio Pagni, Pathology Unit, Desio General Hospital, 20033 Desio, Italy
Gian Luigi Corti, Endoscopy Unit, Division of Surgery, Desio General Hospital, 20033 Desio, Italy
Mara Piatti, Laboratory Medicine Unit, Desio General Hospital, 20033 Desio, Italy
Author contributions: Casella G, Bassotti G and Baldini V ideated and planned the study and wrote the paper; Bassotti G and Sabatino G carried out the statistical analysis; Villanacci V, Di Bella C and Pagni F analyzed the histological samples; Corti GL helped with endoscopic procedures; Piatti M helped with laboratory analyses.
Correspondence to: Gabrio Bassotti, Professor, Gastroenterology and Hepatology Section, Department of Clinical and Experimental Medicine, Ospedale Santa Maria della Misericordia, Piazzale Menghini, 1, 06156 San Sisto (Perugia), Italy. gabassot@tin.it
Telephone: +39-75-5784423 Fax: +39-75-5847570
Received: August 10, 2010 Revised: November 13, 2010
Accepted: November 20, 2010
Published online: June 28, 2011

Abstract

AIM: To investigate whether this might be related to the presence of hyperhomocysteinemia.

METHODS: From January 1998 to December 2008, we evaluated the presence of hyperhomocysteinemia in a series of 165 adult celiac disease (CD) patients (138 females and 27 males, mean age 43 years).

RESULTS: Hyperhomocysteinemia was evident in 32 patients (19.3%), although most of them had moderate levels (mean value 25 mcg/ml; range 15-30). Only one patient had a history of myocardial infarction (heterozygosis for N5-N10-metil tetrahydrofolate reductase mutation).

CONCLUSION: The systematic assessment of hyperhomocysteinemia seems, at present, unjustified in CD patients.

© 2011 Baishideng. All rights reserved.

Key words: Celiac disease; Endoscopy; Histology; Hyperhomocysteinemia

Peer reviewers: Javier San Martín, Chief, Gastroenterology and Endoscopy, Sanatorio Cantegril, Av. Roosevelt y P 13, Punta del Este 20100, Uruguay; Andrew S Day, MB, ChB, MD, FRACP, AGAF, Associate Professor, Department of Paediatrics, University of Otago, Christchurch, PO Box 4345, Christchurch 8140, New Zealand

Casella G, Bassotti G, Villanacci V, Di Bella C, Pagni F, Corti GL, Sabatino G, Piatti M, Baldini V. Is hyperhomocysteinemia relevant in patients with celiac disease? *World J Gastroenterol* 2011; 17(24): 2941-2944 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2941.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2941

INTRODUCTION

Hyperhomocysteinemia, considered as an important risk factor in venous thrombosis[1-3], has a prevalence in the general population of 5%-7%[4], and causes damage of the vascular endothelium by disrupting the release of nitric oxide, an important vasodilator factor[5], followed by

platelet activation and thrombus formation[4].

Celiac disease (CD) is a gluten-sensitive enteropathy due to intolerance to dietary wheat gliadin and related proteins in genetically predisposed individuals[6]. The malabsorption of folates and vitamins (the deficiency of which may be a cause of hyperhomocysteinemia) is frequent in CD patients, either in the classic or oligosymptomatic type[7], and several cases of thrombosis have been reported in patients with CD before establishing a diagnosis of gluten-related duodenal mucosal damage[8,9]. Thus, we investigated the presence of hyperhomocysteinemia in a series of patients with CD, to see whether it might be increased and represent a marker of increased venous thrombosis in these patients.

MATERIALS AND METHODS

Patients

In the period January 1998-December 2008, 165 patients with CD (27 men, 138 women, mean age 43 years) were studied. Inclusion criteria were: positivity for anti-endomysial IgA and anti-transglutaminase IgA antibodies (Eurospital, Trieste, Italy) and duodenal histology suggestive for CD.

Histological assessment

Four samples were obtained by endoscopy forceps from the proximal and distal parts of the duodenum. The biopsies, correctly oriented on acetate cellulose filters (Bio-Optica, Milano, Italy), were fixed in 10% buffered formalin, processed and included in paraffin. After obtaining 5 μm thick sections, these were stained with Hematoxylin-Eosin; some sections were also processed for immunohistochemistry and stained with an anti-CD3 monoclonal antibody (Dako, Denmark) to identify intra-epithelial lymphocytes (IEL). IEL density was expressed as the number of IEL/100 epithelial cells, with a density value of > 25 cells considered as pathological. Histological classification was based on the Marsh-Oberhuber criteria[10] and a new, recently proposed simpler classification[11,12] (Table 1).

Table 1 The Marsh-Oberhuber classification of duodenal histological lesions in celiac disease, compared to the "simplified classification"[11,12]

Histologic type	IEL	Glandular crypts	Villi	Simplified
0	Normal (< 25/100 epithelial cells)	Normal	Normal	Normal
1	Increased	Normal	Normal	Grade A
2	Increased	Hyperplastic	Normal	Grade A
3a	Increased	Hyperplastic	Mild atrophy	Grade B1
3b	Increased	Hyperplastic	Moderate atrophy	Grade B1
3c	Increased	Hyperplastic	Severe (total) atrophy	Grade B2

IEL: Intra-epithelial lymphocytes.

Laboratory assessment

Serum homocysteinemia, vitamin B12 and folic acid levels were measured in all patients. In case of hyperhomocysteinemia, mutations in N5-N10-metil tetrahydrofolate reductase (MTHFR), cystathionine beta synthetase (CBS) and the prothrombin gene were searched for. DNA was extracted from whole blood collected in tubes containing K3-EDTA using a commercial kit (Genomic DNA Isolation kit, Puregene -Gentra System). DNA analysis for MTHFR gene mutation (C677T) was performed by a PCR-RFLP method, as previously described[13]. A fragment of 232 base pairs was then amplified by polymerase chain reaction. The fragment was digested by *Hin*f I restriction enzyme, and subsequent electrophoresis on ethidium bromide stained 3% agarose gel was performed.

The concentration of total homocysteine in plasma (K3-EDTA tubes) was determined by high performance liquid chromatography, as previously described[14]. Basal hyperhomocysteinemia (normal value 5-15 μmol/L) was classified as moderate (16-30 μmol/L), intermediate (31-100 μmol/L) and severe (> 100 μmol/L) according to Hankey *et al*[15]. In all patients, the presence of any thrombotic episode was also evaluated.

The study was approved by the Institutional Review Board of the Desio Hospital.

RESULTS

Histological findings

Most CD patients (24/32, 75.0%) showed mild to severe villous atrophy, with the latter being present in 41.0% of patients (Table 2).

Laboratory findings

Overall, hyperhomocysteinemia was detected in 32 (19.4%) CD patients (24 women, 8 men); average symptoms' onset was 7 (range 1-40) years. Table 3 shows the serologic findings of these patients. Most patients (29/32, 91.0%) had moderate hyperhomocysteinemia, two (6.0%) intermediate and one (3.0%) severe increase of this value. Mutation of MTHFR was found in 13 (41.0%) patients, 7 homozygotes and 6 heterozygotes; one patient displayed heterozygotic mutation of the prothrombin gene. No CBS mutations were found.

Serum B12 vitamin levels were low in 5 (15.6%) patients and serum folate levels were low in 6 (19.0%) patients. No correlation (Spearman's test) was found between serum homocysteine and age (r = 0.10, P = 0.58), gender (r = 0.66, P = 0.07), onset of symptoms (r = -0.06, P = 0.75), vitamin B12 (r = -026, P = 0.14), folic acid (r = 0.05, P = 0.75), and histological grading (r = -0.01, P = 0.9). Moreover, no correlation was also found between histological grading, vitamin B12 (r = -0.10, P = 0.56) and folic acid (r = -0.2, P = 0.3) values.

Clinical findings

Concerning vascular pathology, one patient with heterozygosis for MTHFR mutation and moderate hyperhomocysteinemia had myocardial infarction, whereas the single

Table 2 Demographic, histological findings and associated diseases of 32 celiac disease patients with hyperhomocysteinemia

No.	Sex/age (yr)	Histology (Marsh/simplified classification)	Associated diseases
1	F/37	Marsh 2 (Grade A)	IgA deficit
2	F/38	Marsh 3a (Grade B1)	
3	M/34	Marsh 3a (Grade B1)	
4	F/32	Marsh 3c (Grade B2)	
5	F/20	Marsh 3c (Grade B2)	
6	F/47	Marsh 3b (Grade B1)	
7	F/64	Marsh 1 (Grade A)	
8	F/41	Marsh 3c (Grade B2)	Epilepsy
9	F/22	Marsh 2 (Grade A)	IgA deficit
10	F/61	Marsh 3a (Grade B1)	
11	M/44	Marsh 3c (Grade B2)	NASH
12	F/35	Marsh 3c (Grade B2)	type 1 diabetes
13	F/39	Marsh 3c (Grade B2)	
14	F/40	Marsh 3c (Grade B2)	
15	F/56	Marsh 3c (Grade B2)	PBC
16	F/35	Marsh 3c (Grade B2)	
17	F/42	Marsh 2 (Grade A)	
18	M/33	Marsh 3b (Grade B1)	Sarcoidosis
19	F/31	Marsh 3a (Grade B1)	
20	F/42	Marsh 3a (Grade B1)	
21	M/28	Marsh 3a (Grade B1)	
22	F/41	Marsh 3c (Grade B2)	
23	F/45	Marsh 3c (Grade B2)	
24	F/21	Marsh 3a (Grade B1)	
25	F/29	Marsh 3b (Grade B1)	
26	F/55	Marsh 3a (Grade B1)	Osteoporosis
27	F/78	Marsh 3c (Grade B2)	
28	M/47	Marsh 3c (Grade B2)	
29	M/63	Marsh 3b (Grade B1)	Psoriasis, myocardial infarction
30	M/18	Marsh 3a (Grade B1)	
31	M/40	Marsh 1 (Grade A)	
32	F/32	Marsh 3a (Grade B1)	Sarcoidosis

IgA: Immunoglobulin A; PBC: Primary biliary cirrhosis.

Table 3 Serologic findings of 32 celiac disease patients with hyperhomocysteinemia

No.	Serum homocysteine (NV 5-15 μmol/L)	Serum B12 vitamin (NV 190-66 pg/mL)	Serum folic acid (NV 2-14 ng/mL)	Genetic mutation
1	14	365	10	
2	13.5	267	4.7	
3	15	369	6	
4	20	356	1	
5	13	174	5	MTHFR (het)
6	21	311	1.5	
7	15	354	12	
8	44	293	5.2	MTHFR (hom)
9	15	493	6	MTHFR (hom)
10	17	333	1	MTHFR (het)
11	19	383	3	
12	17	198	2	
13	15	400	3	MTHFR (het)
14	27	720	4	MTHFR (hom)
15	13	699	3	MTHFR (hom)
16	21	246	2.4	
17	18	265	5.1	
18	19	282	1	Prothr (het)
19	20	457	3	MTHFR (hom)
20	14	555	0.5	
21	13	291	2	
22	23	280	6	
23	14	329	1	MTHFR (het)
24	17	684	10	
25	19	566	5.6	
26	20	188	2	
27	20.5	154	3	
28	20.5	216	2	MTHFR(het)
29	16	164	13	MTHFR (het)
30	25	555	8	
31	149	150	2	MTHFR (hom)
32	31	385	2	MTHFR (hom)

het: Heterozygosis; hom: Homozygosis; MTHFR: N5-N10-metil tetrahydrofolate reductase.

patient with severe hyperhomocysteinemia underwent coronary angiography for atypical chest pain, but no evidence of vessel pathology was found. No patient in this series had episodes of venous or arterial thrombosis, or any stroke episodes.

DISCUSSION

Our findings show that hyperhomocysteinemia is relatively frequent in patients with CD, being present in about 20% of the patients in our series. Hyperhomocysteinemia might represent a link between undiagnosed gluten-sensitive enteropathy and some of its complications[16]. Interestingly, these results were similar to those obtained in an overlapping geographic area, which showed the presence of hyperhomocysteinemia in about 20% of newly diagnosed CD patients compared to about 6% of controls[17].

Hyperhomocysteinemia may be due to genetic factors, with CBS deficiency being considered the most common genetic cause[5,17], or from acquired folate and vitamin B12 deficiencies[18,19]. A homozygous deficiency of MTHFR, the vitamin B12 dependent enzyme for the remethylation of homocysteine to methionine, may cause hyperhomocysteinemia and it has a worse prognosis than CBS deficiency for the absence of an effective therapy[20]. Moreover, treatment with a gluten-free diet and folic acid in CD patients with MTHFR variants does not consistently improve hyperhomocysteinemia[21].

Thus, CD (in which malabsorption of folate and vitamin B12 is common[22]) might lead to increased cardiovascular risks due to an increase of secondary (acquired) hyperhomocysteinemia, further aggravated by the possible presence of genetic abnormalities responsible for hyperhomocysteinemia. However, notwithstanding the relative frequency of hyperhomocysteinemia in our CD patients, this was almost always of moderate entity, with only one patient displaying high levels. Interestingly, the only patient to have a cardiovascular event (myocardial infarction) had relatively low levels of hyperhomocysteinemia and presented heterozygous mutations of MTHFR. No CBS mutations were found in our series. Only one mutation of the prothrombin gene was found, and this is in line with the paucity of reports of such mutations in CD patients[23].

In conclusion, at present it seems unnecessary to systematically investigate CD for the presence of hyperhomocysteinemia; conversely, a serological screening for CD in patients with hyperhomocysteinemia, cardiovascular events and vitamin deficiency could be considered, especially because adult CD patients may display only a few to no intestinal symptoms[24,25], and the onset of the disease may rarely be due to a thrombotic event[26-28].

COMMENTS

Background

Venous thrombosis has been reported in patients with celiac disease (CD). Since this might be related to hyperhomocysteinemia, a risk factor for vascular disease, we investigated the prevalence of hyperhomocysteinemia in a series of adult celiac patients.

Research frontiers

An increased prevalence of hyperhomocysteinemia in CD might lead to increased cardiovascular risk.

Innovations and breakthroughs

To date, most data on this topic originates from single reports, and only one other study investigated systematically celiac patients.

Applications

It appears that, given the low prevalence of hyperhomocysteinemia in celiac patients, it is unnecessary to screen systematically patients; this is useful information in terms of sanitary expenses.

Peer review

The authors evaluated in a cohort of 165 CD patients the presence of hyperhomocysteinemia during a period of time of 10 years. They showed that seems unnecessary to investigate systematically CD for the presence of hyperhomocysteinemia. Their work could contribute to the epidemiologic information of the CD in the Italian population.

REFERENCES

1 **Nygård O**, Vollset SE, Refsum H, Brattström L, Ueland PM. Total homocysteine and cardiovascular disease. *J Intern Med* 1999; **246**: 425-454

2 **Simioni P**, Prandoni P, Burlina A, Tormene D, Sardella C, Ferrari V, Benedetti L, Girolami A. Hyperhomocysteinemia and deep-vein thrombosis. A case-control study. *Thromb Haemost* 1996; **76**: 883-886

3 **den Heijer M**, Koster T, Blom HJ, Bos GM, Briet E, Reitsma PH, Vandenbroucke JP, Rosendaal FR. Hyperhomocysteinemia as a risk factor for deep-vein thrombosis. *N Engl J Med* 1996; **334**: 759-762

4 **Welch GN**, Loscalzo J. Homocysteine and atherothrombosis. *N Engl J Med* 1998; **338**: 1042-1050

5 **Lim PO**, Tzemos N, Farquharson CA, Anderson JE, Deegan P, MacWalter RS, Struthers AD, MacDonald TM. Reversible hypertension following coeliac disease treatment: the role of moderate hyperhomocysteinaemia and vascular endothelial dysfunction. *J Hum Hypertens* 2002; **16**: 411-415

6 **Armstrong MJ**, Robins GG, Howdle PD. Recent advances in coeliac disease. *Curr Opin Gastroenterol* 2009; **25**: 100-109

7 **Bai JC**. Malabsorption syndromes. *Digestion* 1998; **59**: 530-546

8 **Hida M**, Erreimi N, Ettair S, Mouane N, Bouchta F. [Associated celiac disease and venous thrombosis]. *Arch Pediatr* 2000; **7**: 215-216

9 **Gabrielli M**, Santoliquido A, Gasbarrini G, Pola P, Gasbarrini A. Latent coeliac disease, hyperhomocysteinemia and pulmonary thromboembolism: a close link? *Thromb Haemost* 2003; **89**: 203-204

10 **Oberhuber G**, Granditsch G, Vogelsang H. The histopathology of coeliac disease: time for a standardized report scheme for pathologists. *Eur J Gastroenterol Hepatol* 1999; **11**: 1185-1194

11 **Corazza GR**, Villanacci V. Coeliac disease. *J Clin Pathol* 2005; **58**: 573-574

12 **Corazza GR**, Villanacci V, Zambelli C, Milione M, Luinetti O, Vindigni C, Chioda C, Albarello L, Bartolini D, Donato F. Comparison of the interobserver reproducibility with different histologic criteria used in celiac disease. *Clin Gastroenterol Hepatol* 2007; **5**: 838-843

13 **Kluijtmans LA**, van den Heuvel LP, Boers GH, Frosst P, Stevens EM, van Oost BA, den Heijer M, Trijbels FJ, Rozen R, Blom HJ. Molecular genetic analysis in mild hyperhomocysteinemia: a common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. *Am J Hum Genet* 1996; **58**: 35-41

14 **Accinni R**, Campolo J, Bartesaghi S, De Leo G, Lucarelli C, Cursano CF, Parodi O. High-performance liquid chromatographic determination of total plasma homocysteine with or without internal standards. *J Chromatogr A* 1998; **828**: 397-400

15 **Hankey GJ**, Eikelboom JW. Homocysteine and vascular disease. *Lancet* 1999; **354**: 407-413

16 **Saibeni S**, Lecchi A, Meucci G, Cattaneo M, Tagliabue L, Rondonotti E, Formenti S, De Franchis R, Vecchi M. Prevalence of hyperhomocysteinemia in adult gluten-sensitive enteropathy at diagnosis: role of B12, folate, and genetics. *Clin Gastroenterol Hepatol* 2005; **3**: 574-580

17 **Malinowska A**, Chmurzynska A. Polymorphism of genes encoding homocysteine metabolism-related enzymes and risk for cardiovascular disease. *Nutr Res* 2009; **29**: 685-695

18 **Cravo ML**, Glória LM, Selhub J, Nadeau MR, Camilo ME, Resende MP, Cardoso JN, Leitão CN, Mira FC. Hyperhomocysteinemia in chronic alcoholism: correlation with folate, vitamin B-12, and vitamin B-6 status. *Am J Clin Nutr* 1996; **63**: 220-224

19 **Cravo ML**, Camilo ME. Hyperhomocysteinemia in chronic alcoholism: relations to folic acid and vitamins B(6) and B(12) status. *Nutrition* 2000; **16**: 296-302

20 **Mudd SH**, Levy HL, Skovby F. Disorders of transsulfuration. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The Metabolic and Molecular Basis of Inherited Disease. 7th ed. New York, NY: McGraw-Hill, 1995: 1279-1327

21 **Wilcox GM**, Mattia AR. Celiac sprue, hyperhomocysteinemia, and MTHFR gene variants. *J Clin Gastroenterol* 2006; **40**: 596-601

22 **Halfdanarson TR**, Litzow MR, Murray JA. Hematologic manifestations of celiac disease. *Blood* 2007; **109**: 412-421

23 **Gould J**, Deam S, Dolan G. Prothrombin 20210A polymorphism and third generation oral contraceptives--a case report of coeliac axis thrombosis and splenic infarction. *Thromb Haemost* 1998; **79**: 1214-1215

24 **Setty M**, Hormaza L, Guandalini S. Celiac disease: risk assessment, diagnosis, and monitoring. *Mol Diagn Ther* 2008; **12**: 289-298

25 **Rubio-Tapia A**, Murray JA. Celiac disease. *Curr Opin Gastroenterol* 2010; **26**: 116-122

26 **Kremer Hovinga JA**, Baerlocher G, Wuillemin WA, Solenthaler M. [Deep venous thrombosis of the leg in acquired thrombophilia--hyperhomocysteinemia as a sequela of undetected celiac disease]. *Ther Umsch* 1999; **56**: 519-522

27 **Audia S**, Duchêne C, Samson M, Muller G, Bielefeld P, Ricolfi F, Giroud M, Besancenot JF. [Stroke in young adults with celiac disease]. *Rev Med Interne* 2008; **29**: 228-231

28 **Baryshnikov EN**, Krums LM, Vorob'eva NN, Parfenov AI. [Lower extremity deep vein thrombosis associated with gluten-sensitivity celiac disease]. *Ter Arkh* 2010; **82**: 52-54

S- Editor Tian L **L- Editor** Rutherford A **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2945

World J Gastroenterol 2011 June 28; 17(24): 2945-2952
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2011 Baishideng. All rights reserved.

BRIEF ARTICLE

Long-term effects of lamivudine treatment in Japanese chronic hepatitis B patients

Masayuki Murata, Norihiro Furusyo, Mami Unno, Eiichi Ogawa, Kazuhiro Toyoda, Hiroaki Taniai, Hachiro Ohnishi, Jun Hayashi

Masayuki Murata, Norihiro Furusyo, Mami Unno, Eiichi Ogawa, Kazuhiro Toyoda, Hiroaki Taniai, Hachiro Ohnishi, Jun Hayashi, Department of General Internal Medicine, Kyushu University Hospital, Fukuoka, 812-8582, Japan

Norihiro Furusyo, Mami Unno, Jun Hayashi, Department of Environmental Medicine and Infectious Disease, Faculty of Medical Sciences, Kyushu University, Fukuoka, 812-8582, Japan

Author contributions: Murata M and Furusyo N performed the majority of the experiments; Unno M, Ogawa E, Toyoda K, Taniai H and Ohnishi H provided clinical serum samples from patients for the study; Murata M and Furusyo N designed the study; Murata M analyzed data and wrote the manuscript; Hayashi J reviewed the manuscript in addition to providing financial support for this study.

Correspondence to: Masayuki Murata, MD, PhD, Department of General Internal Medicine, Kyushu University Hospital, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan. mmurata@gim.med.kyushu-u.ac.jp

Telephone: +81-92-6425909 Fax: +81-92-6425916

Received: August 28, 2010 Revised: February 15, 2011
Accepted: February 22, 2011
Published online: June 28, 2011

Abstract

AIM: To analyze the association between the emergence of tyrosine-methionine-asparatate-asparatate (YMDD) mutants (reverse transcription; rtM204I/V) and deterioration of liver function during long-term lamivudine treatment of Japanese patients with chronic hepatitis B virus (HBV) infection.

METHODS: The data of 61 consecutive Japanese patients with chronic hepatitis B who underwent continuous lamivudine treatment for more than 24 mo and had a virological response were analyzed. Analysis of YMDD mutants was done by real-time polymerase chain reaction with LightCycler probe hybridization assay for up to 90 mo (mean, 50.8 mo; range, 24-90 mo).

RESULTS: A mixed mutant-type (YMDD + tyrosine-isoleucine-asparatate-asparatate: YIDD or tyrosine-valine-asparatate-asparatate: YVDD) or a mutant-type (YIDD or YVDD) were found in 57.4% of 61 patients at 1 year, 78.7% of 61 patients at 2 years, 79.6% of 49 patients at 3 years, 70.5% of 34 patients at 4 years, 68.4% of 19 patients at 5 years, 57.1% of 14 patients at 6 years, and 33.3% of 6 patients at 7 years. Of the 61 patients, 56 (92%) had mixed mutant- or a mutant-type. Only 5 (8%) had no mutants at each observation point. Virological breakthrough was found in 26 (46.4%) of 56 patients with YMDD mutants, 20 of whom had a hepatitis flare-up: the remaining 30 (53.6%) had neither a virological breakthrough nor a flare-up. All 20 patients who developed a hepatitis flare-up had a biochemical and virological response after adefovir was added to the lamivudine treatment.

CONCLUSION: Our results suggest that it is possible to continue lamivudine treatment, even after the emergence of YMDD mutants, up to the time that the patients develop a hepatitis flare-up.

© 2011 Baishideng. All rights reserved.

Key words: Tyrosine-methionine-asparatate-asparatate mutant; Hepatitis B virus; Lamivudine; Drug resistance

Peer reviewers: Nageshwar D Reddy, Professor, Asian Institute of Gastroenterology, 6-3-652, Somajiguda, Hyderabad 500 082, India; Hanna Gregorek, PhD, Assistant Professor, Department of Microbiology and Clinical Immunology, The Children's Memorial Health Institute, Al. Dzieci Polskich 20, Warsaw 04-730, Poland

Murata M, Furusyo N, Unno M, Ogawa E, Toyoda K, Taniai H, Ohnishi H, Hayashi J. Long-term effects of lamivudine treatment in Japanese chronic hepatitis B patients. *World J Gastroenterol* 2011; 17(24): 2945-2952 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2945.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2945

INTRODUCTION

Chronic hepatitis B virus (HBV) infection affects more than 350 million people worldwide, with 75% living in the Asia-Pacific region[1,2]. Although Japan was historically endemic for HBV infection, our previous studies have shown that the prevalence of hepatitis B surface antigen (HBsAg) carriage in Okinawa, Japan markedly decreased from 12.4% in 1970 to 4.2% in 1996[3,4]. However, chronic HBV infection continues to be a major health problem because it leads to the development of liver cirrhosis, hepatocellular carcinoma (HCC), and raises the risk of hepatic disease-related death. Because HBV replication is associated with liver injury, therapy for patients with chronic HBV infection aims to stop or reduce disease progression and to prevent the development of hepatic decompensation through the sustained suppression of HBV replication[5]. For this purpose, interferon or oral antiviral nucleos(t)ide analogues, such as lamivudine, adefovir, entecavir, telbivudine and tenofovir have been approved for the treatment of patients with chronic HBV infection. Previous studies have shown that HBV genotype influences liver disease progression[6,7], and our epidemiological study of the Japanese HBV genotype distribution showed that most patients were infected with genotype C[8]. Because genotype C has been reported to be associated with severe liver damage and to be resistant to non-pegylated interferon[8-10], Japanese patients with chronic hepatitis B are often given nucleos(t)ide analog treatment. Nucleos(t)ide analogs have fast and potent inhibitory effects on HBV polymerase and reverse transcriptase activity, are safe and easy to use, and can induce HBV DNA suppression, alanine aminotransferase (ALT) normalization and improvement of liver histology[11-14].

Lamivudine is the first oral nucleoside analogue to be approved for the treatment of chronic hepatitis B patients, and it has been shown to suppress HBV replication by interfering with HBV DNA polymerase and disease activity, to reduce the incidence of HCC, and to improve survival[15,16]. A study that included a large number of Japanese chronic hepatitis B patients showed lamivudine to have good virological and biochemical efficacy in long-term treatment[17].

Although there is much evidence supporting the effectiveness of lamivudine for chronic hepatitis B patients, the clinical benefit of lamivudine treatment has been eroded, in the case of long-term treatment, by the emergence of lamivudine-resistant HBV mutants with mutation of the reverse transcriptase domain of the polymerase gene. The emergence of lamivudine-resistant mutants is mainly based on point mutation from methionine to valine/isoleucine at rt204 (rt204V/I) in the tyrosine-methionine-asparatate-asparatate (YMDD) motif[18]. The emergence of lamivudine-resistant HBV has been linked to virological breakthrough, sometimes followed by biochemical breakthrough, and to flare-ups of hepatitis[19].

The detection of YMDD mutants has mainly been done by methods such as direct DNA sequencing or hybridization[20,21], but these methods are labor-intensive and time-consuming. Fluorometric real-time polymerase chain reaction (PCR) with the LightCycler probe hybridization assay is reported to be an easy, rapid and accurate method for the detection of YMDD mutants[22,23]. Few studies have sequentially assessed the emergence of YMDD mutants during long-term lamivudine treatment in Japan. The aim of the present study was to analyze the association between the emergence of YMDD mutants and deterioration of liver function during long-term lamivudine treatment of Japanese chronic hepatitis B patients by use of the LightCycler probe hybridization assay.

MATERIALS AND METHODS

Patients

The study included 61 consecutive Japanese patients with chronic hepatitis B who underwent continuous lamivudine treatment for more than 24 mo, and had a virological response (defined as a decline of more than 4.0 log copies/mL in HBV DNA level during treatment). The patients started lamivudine treatment between February 2001 and May 2007 at the Department of General Internal Medicine, Kyushu-University Hospital in Fukuoka, Japan. Before the start of lamivudine treatment, all patients had HBsAg and detectable levels of HBV DNA by PCR assay. The diagnosis of chronic hepatitis and cirrhosis was based on a liver biopsy for most patients, but if unavailable it was based on clinical laboratory and ultrasonography data. None of the patients tested positive for antibody to hepatitis C virus or human immunodeficiency virus type 1, nor was there evidence of other forms of liver diseases, such as alcoholic liver disease, drug-induced liver disease, or autoimmune hepatitis.

All patients received lamivudine (Zeffix, Glaxo Smith Kline, UK) in a single oral daily dose of 100 mg. Observation was for up to 90 mo (mean, 50.8 mo; range, 24-90 mo) after the start of lamivudine administration, and the emergence of YMDD mutants during treatment was identified using the LightCycler probe hybridization assay. Serum ALT, hepatitis B e antigen (HBeAg), anti-HBe, and HBV DNA were measured every 1-2 mo. Sera were tested for mutation of the HBV polymerase gene every 6-12 mo during treatment.

Definitions of "virological breakthrough" and "flare-up" of hepatitis

Virological breakthrough was defined as an increase in serum HBV DNA of more than 1 log copies/mL from the nadir of the initial response[19]. A flare-up of hepatitis was defined as an increase in ALT level to more than 3 times the upper limit of normal.

Routine laboratory tests and viral markers

Biochemical tests were performed using standard procedures before and at least once monthly during treatment. HBsAg, HBeAg and anti-HBe were determined by a chemiluminescence enzyme immunoassay (Abbott Japan Co., Tokyo, Japan). HBV genotype analysis was performed by

a previously reported method[24]. Serum HBV DNA level was measured by a PCR-based method (Roche Amplicor HBV Monitor; Roche Diagnostics, Mannheim, Germany). The detection range of the assay was between 2.6 (corresponding to 400 copies/mL) and 8.7 log copies/mL.

Serum samples and extraction of HBV DNA

Serum samples were obtained from all patients before and during lamivudine treatment and stored at -20℃ until use. HBV DNA was extracted from serum using the QIAamp DNA mini kit (Qiagen Ltd., Crawley, United Kingdom) according to the manufacturer's instructions.

Monitoring the emergence of lamivudine-resistant mutants by the LightCycler probe hybridization assay

Lamivudine-resistant mutation was detected by rapid PCR amplification across the YMDD-encoding gene locus and analysis of the hybridization kinetics of an integrated probe to infer its sequence was done using the LightCycler (Roche Diagnostics) according to the method reported by Whalley *et al*[22].

Briefly, HBV DNA was extracted from serum, and a 399 bp region of the polymerase gene was amplified by hemi-nested PCR assay. The amplified PCR product was denatured and hybridized to the Bi-probe system, which uses Cy5-labeled probes in conjunction with SYBR Green I (Bio/Gene, Kimbolton, United Kingdom). The LightCycler was used for amplification of PCR clones and to determine the melting characteristics of the probe-amplification hybrid. A melting curve analysis of the data was performed using the LightCycler analysis software v3.5 (Roche Diagnostics). Melting curves were converted to melting peaks by plotting the negative derivative of fluorescence with respect to temperature (-dF/dT). This analysis gave the melting temperature (*Tm*) at which 50% of the hybridizing probe was annealed to the PCR product. Because the presence of a single-base mismatch results in a shift in the melting temperature to a temperature lower than that for the probe-specific sequence, analysis of the probe melting curves allows differentiation of the wild-type YMDD from the YMDD mutants including YIDD, YVDD, and the YMDD/YIDD and YMDD/YVDD mixed types. The detection limit of this assay was about 10%-20% of the total virus pool (data not shown).

Statistical analysis

The distribution of continuous variables was analyzed by the Mann-Whitney *U* test. Differences in proportions were tested by Fisher's exact test. A two-tailed *P* value of less than 0.05 was considered statistically significant. Statistical analysis was performed with the SPSS statistical package (version 11.0, SPSS, Inc., Chicago, IL, USA).

RESULTS

Baseline characteristics

Of the 61 patients, 45 were male with a median age of 50 years (range, 28-65 years) and 16 female with a median age of 49 years (range, 38-69 years). All patients were infected with genotype C, 36 (59%) were negative for HBeAg, 15 (25%) were cirrhotic, and the median HBV DNA level was 6.5 log copies/mL (range, 2.7-8.7 log copies/mL).

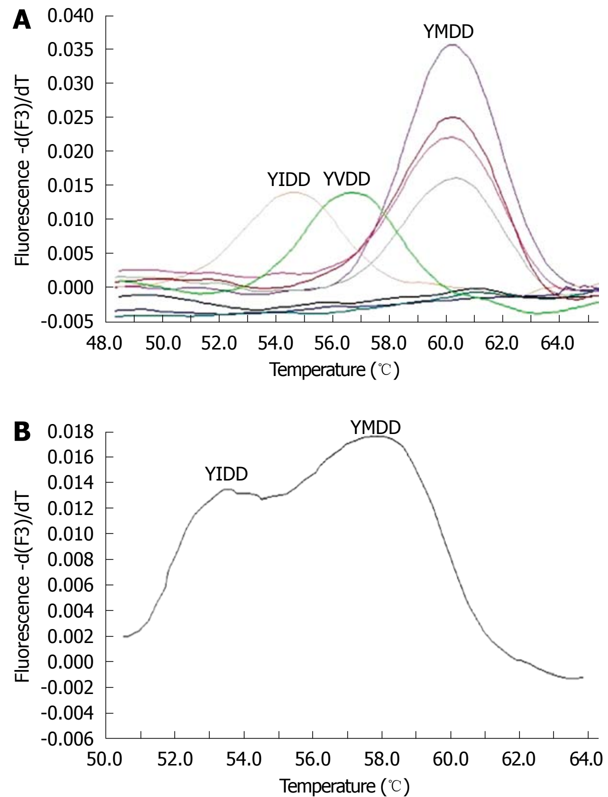


Figure 1 Melting curve analysis using the LightCycler probe hybridization assay. The melting curves were converted to melting peaks by plotting the negative derivative of fluorescence with respect to temperature [-d(F3)/dT]. The melting temperature (*Tm*) for each of the YMDD mutants is indicated with a vertical line. A: Representative results for differentiating HBV YMDD mutants. YMDD, YVDD and YIDD mutants showed distinct *Tm* values; B: The shoulder curve indicated the mixed mutant-type. Representative results show YMDD + YIDD mutants.

Melting curve analysis for the detection of HBV YMDD mutants

YMDD mutants were analyzed by melting curve analysis. The melting peaks of the wild-type and mutant HBV strains were typically observed at different temperatures, as shown in Figure 1A. The melting temperatures of the YIDD and YVDD mutants were approximately 9℃ and 2.5℃ lower than that of the wild-type, respectively. Because the melting curve showed a double peak in the case of YIDD or YVDD mutant mixed with the wild-type YMDD, as shown in Figure 1B, this type of melting curve was considered to be the mixed mutant-type.

Seven-year change in the wild, mixed mutant and mutant-types

The mixed mutant- or mutant-type was found in 57.4% of 61 patients at 1 year, 78.7% of 61 patients at 2 years,

Table 1 Comparison of patients with and without the emergence of YMDD mutants during treatment, median (range)

Characteristics	Mutant- or mixed mutant-type (n = 56)	Not detected or wild-type (n = 5)	P-value
No. of men (%)	42 (75.0)	3 (80.0)	NS
Age (yr)	50 (28-69)	52 (34-55)	NS
ALT level (U/L)	79 (15-1593)	63 (44-108)	NS
Albumin (g/dL)	4.1 (2.9-5.0)	4.3 (4.0-4.3)	NS
Platelet count (× 10^4/mL)	13.1 (3.3-43.3)	17.8 (15.2-26.0)	NS
HBeAg positivity (%)	23 (41.1)	2 (40.0)	NS
HBV-DNA level (log copies/mL)	6.5 (2.7-8.7)	7.0 (5.4-8.7)	NS
Cirrhosis (%)	22 (39.3)	1 (20.0)	NS
History of HCC (%)	8 (14.3)	1 (20.0)	NS
Virological breakthrough (%)	26 (46.4)	0	< 0.0001
Patients with hepatitis flare-ups (%)	20 (35.7)	0	< 0.0001

Between group comparison was done of whether or not YMDD mutant was detected at each observation point. ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; NS: Not significant.

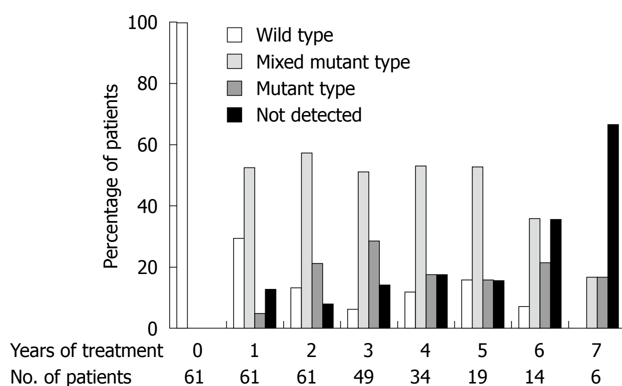


Figure 2 Change in the proportion of YMDD wild, mixed mutant, and mutant-types during lamivudine treatment.

79.6% of 49 patients at 3 years, 70.5% of 34 patients at 4 years, 68.4% of 19 patients at 5 years, 57.1% of 14 patients at 6 years, 33.3% of 6 patients at 7 years, as shown in Figure 2.

Comparison of patients with and without the emergence of YMDD mutants during treatment

Of the 61 patients, 56 (92%) had the mixed mutant- or mutant-type: Only 5 (8%) had none of the tested mutations during the observation period. Virological breakthrough or a flare-up of hepatitis was observed in 26 (46.4%) and 20 (35.7%), respectively, of the 56 patients with YMDD mutants. None of the 5 patients without YMDD mutants had virological breakthrough or a flare-up of hepatitis. Virological breakthrough or a flare-up of hepatitis was experienced significantly more often by patients with than without YMDD mutants (P < 0.0001).

No significant differences in sex, age, pretreatment ALT level, serum albumin, platelet count, frequency of HBeAg positivity, pretreatment HBV DNA level, presence of cirrhosis, or history of HCC were observed between these groups. Of the 56 patients with YMDD mutants, 30 (53.6%) had no virological breakthrough. However, no significant differences in sex, age, pretreatment ALT level, serum albumin, platelet count, frequency of HBeAg positivity, pretreatment HBV DNA level, presence of cirrhosis, or history of HCC were observed for the patients with mutants, with or without virological breakthrough (data not shown) (Table 1).

Table 2 Comparison of the emergence of YMDD mutants during treatment by hepatitis B e antigen status of patients, median (range)

Characteristics	HBeAg negative (n = 36)	HBeAg positive (n = 25)	P-value
No. of men (%)	28 (78)	17 (68)	NS
Age (yr)	50 (29-69)	50 (28-60)	NS
HBV DNA level (log copies/mL)	5.8 (2.7-7.6)	7.6 (4.1-8.7)	< 0.0001
Patients with YMDD mutants (%)	33 (92)	23 (92)	NS
Virological breakthrough (%)	12 (33)	14 (56)	NS
Patients with flare-ups of hepatitis (%)	9 (25)	11 (44)	NS

HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; NS: Not significant.

Of the 61 patients, 36 (59%) were HBeAg negative. Although about 90% of patients with or without HBeAg had YMDD mutants, HBeAg negative patients had a tendency to have a lower frequency of virological breakthrough and hepatitis flare-ups than HBeAg positive patients. However, no significant between group differences were found in sex, age, number of patients with YMDD mutants, the frequency of virological breakthrough and hepatitis flare-ups; only pretreatment HBV DNA level showed a significant difference (Table 2).

Characteristics of the patients with hepatitis flare-up

Of the 61 patients, a flare-up of hepatitis was experienced by 20 (32.8%), 15 (75%) males, 5 (25%) females, median age 56 years (range, 44-65 years), 11 (55%) with cirrhosis, and 14 (70%) with HBeAg. All patients who developed flare-ups of hepatitis following an increase in the HBV DNA level had YMDD mutation. The peak HBV DNA level (median 6.7 log copies/mL; range, 5.7-8.0 log copies/mL) at the time of a flare-up was significantly lower than at pretreatment (median 7.6 log copies/mL; range, 6.0-8.7 log copies/mL) (P < 0.05) (Table 3).

Clinical course of patients with hepatitis flare-up

All 20 patients who had a flare-up were prescribed 10 mg of adefovir dipivoxil daily, in addition to lamivudine treatment, and all had a biochemical and virological response.

DISCUSSION

Early detection and monitoring of HBV genotypic resis-

Table 3 Characteristics of the patients with flare-ups of hepatitis

Patient	Age (yr)	Sex	Cirrhosis	HBeAg	Change of HBV DNA level after treatment (log copies/mL)			Mutant type
					Pre-treatment	Nadir	Virological breakthrough with hepatitis flare-ups	
1	54	F	+	+	8.7	4.7	7.6	YIDD/YVDD
2	56	M	+	+	8.1	4.1	7.9	Mixed
3	59	M	+	-	6.6	< 2.6	6.8	YIDD
4	60	M	+	+	7.9	< 2.6	8.0	Mixed
5	65	M	+	+	6.5	< 2.6	6.7	Mixed
6	49	M	+	+	7.9	3.9	7.0	YIDD
7	58	M	-	+	8.7	3.9	7.2	YVDD
8	51	M	-	+	7.8	3.8	7.6	YIDD/YVDD
9	58	M	-	+	8.7	4.3	7.9	Mixed
10	61	M	+	-	6.0	< 2.6	6.6	Mixed
11	51	F	-	+	7.2	< 2.6	5.9	YIDD
12	62	F	-	+	7.7	< 2.6	6.6	Mixed
13	44	M	+	+	6.7	< 2.6	5.9	YVDD
14	56	M	-	+	8.7	5.0	7.0	YVDD
15	50	F	-	+	7.6	5.6	6.2	YIDD
16	47	M	+	+	7.9	3.9	6.4	YIDD
17	59	F	+	-	6.9	< 2.6	6.1	YVDD
18	45	M	-	-	6.9	< 2.6	6.1	Mixed
19	61	M	-	-	6.7	< 2.6	5.9	Mixed
20	56	M	+	-	6.6	< 2.6	5.7	YVDD

HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

tance in patients with chronic hepatitis B using nucleoside analogues allows clinicians to avoid virological breakthroughs followed by flare-ups of hepatitis. In this retrospective study, we found that the LightCycler probe hybridization assay was useful for monitoring the emergence of YMDD mutants. Furthermore, our results provide important data on the YMDD mutation of Japanese chronic hepatitis B patients undergoing long-term lamivudine treatment.

Continuous lamivudine treatment is associated with an increased percentage of patients with YMDD mutants. Lamivudine-genotypic resistance was reported in 24% of patients after 1 year of treatment, 41% after 2 years, 53% after 3 years and 70% after 4 years by PCR using a restriction fragment-length polymorphism assay (PCR-RFLP)[25]. Similarly, the present study showed an increased percentage of patients with YMDD mutants within 3 years of treatment, although the percentage was higher than that of the previous report, probably a reflection of different sensitivities of the assay used for the detection of YMDD mutants. Direct DNA sequencing, PCR-RFLP, and reverse hybridization line probe assay are common methods of detecting lamivudine-genotypic resistance[20]. The detection limits of these assays are about 20%, 5%, and down to 10% of the total viral pool, respectively[21]. These methods are labor-intensive, time-consuming, and have the risk of contamination because they require s separate set of endonuclease reactions for each of the mutants, or a specific probe for each mutant. The LightCycler probe hybridization assay can detect YMDD mutants within 1 h and the risk of carryover contamination is minimal because PCR is performed in a closed glass capillary. The detection limit of this assay is about 10%-20% of the total virus pool, and minor subpopulations can be detected (those constituting about 20% of the total population). Our results may more accurately reflect the actual rates of YMDD mutants than were found in previous reports. Because of the quasi-species nature of HBV, YMDD mutants have been detected in patients with chronic hepatitis B who never received lamivudine treatment using a more sensitive method than our method[26-28]. Unfortunately, the present study could not determine if preexisting mutants prevailed after the initiation of lamivudine treatment because this assay could not detect YMDD mutants before treatment.

The present study showed that virological breakthrough and flare-ups of hepatitis occurred after the emergence of YMDD mutants, as reported previously[29]. Therefore, monitoring ALT and HBV DNA levels after the emergence of YMDD mutants is clinically important for the management of patients treated with lamivudine. It has been reported that a short latency to the emergence of YMDD mutants, mixed type YMDD mutant (YIDD + YVDD type), and a low ALT level in patients with YMDD mutants were associated with virological breakthrough or flare-up of hepatitis[30]. In the present study, 20 (35.4%) of 56 patients with YMDD mutants developed flare-ups after virological breakthrough during the treatment. As reported in previous studies[17,31], our results also showed that a flare-up of hepatitis was frequently observed in patients with cirrhosis, or HBeAg positive patients, which may be related to the more active liver disease of HBeAg positive patients. However, our results showed no virological breakthrough by about half of our patients with YMDD mutants during long-term lamivudine treatment. It is important to consider the prognosis

of the patients who continued lamivudine treatment after the emergence of YMDD mutants. It has been reported that there was no benefit for patients who continued lamivudine treatment after the emergence of YMDD mutants compared with patients who discontinued treatment, based on a comparison of the rates of flare-ups of hepatitis, hepatic decompensation, and HBe seroconversion over a 12-mo period[32]. Another report, however, showed a benefit of long-term lamivudine treatment, for 8 years, in Asian patients with YMDD mutants without advanced disease, who had a lower risk of development of cirrhosis and HCC, a greater reduction of HBV DNA level, and a similar rate of flare-ups of hepatitis compared with untreated patients[33]. Our data from the present study suggests that it is possible to continue lamivudine treatment even after the emergence of YMDD mutants if clinicians note the above risk factors associated with virological breakthrough or flare-ups of hepatitis.

The present study showed that about half of patients with YMDD mutants did not encounter virological breakthrough during long-term lamivudine treatment. It has previously been shown that YMDD mutants (rtM204V or rtM204I) have preexisting polymorphisms in HBV-infected patients because of the quasi-species nature of HBV in infected individuals, and that these mutants appeared randomly in viral populations, which had a replication disadvantage to the YMDD wild-type in the absence of lamivudine[34]. A previous study showed that HBV mutants with mutations in the YMDD motif in patients before treatment would not be selected by lamivudine or induce breakthrough hepatitis[27]. Furthermore, the rtM204V mutant in domain C frequently accompanies rtL180M mutants in domain B[26]. *In vitro* studies showed that rtM204I alone had lower replication competency than rtL180M/rtM204V[35]. These data suggest that the gain of replication capacity of YMDD mutants during lamivudine treatment may be associated with multiple factors, including intrinsic replicative advantages potentially conferred by mutations accumulating outside domain C, the fluctuating environment in which these mutants replicate, and the host immune response.

In the present study, 5 (8%) of 61 patients had no emergence of YMDD mutation during the treatment. Hashimoto *et al*[30] reported that factors associated with YMDD mutants not appearing during 5-year lamivudine therapy for patients with chronic HBV infection are HBeAg negativity, lack of cirrhosis, and high γ GTP level. We were not able to confirm their results because there were too few patients free of YMDD mutants to draw a significant conclusion.

Adding adefovir dipivoxil, which is without cross-resistance to lamivudine, is effective for achieving a virological and biochemical response in patients with lamivudine-resistance[36,37]. The American Association for the study of Liver Disease guidelines on HBV recommend the addiction of a second drug in the event of lamivudine resistance[38,39]. It has been reported for patients with lamivudine resistance that the virological and biochemical response rates were similar between a group being switched to adefovir monotherapy and a group for which adefovir was added to lamivudine treatment, but adefovir resistance more frequently occurred in the patients who had combined adefovir and lamivudine treatment[37,40]. Therefore, the add-on treatment is thought to be superior to switching treatment with regard to the prevention of subsequent multi-drug resistance. The above data supported our result of a biochemical and virological response by all patients who had adefovir added to lamivudine treatment after a flare-up of hepatitis.

In conclusion, no virological breakthrough was observed in about half of the patients with YMDD mutants during long-term lamivudine treatment. Patients who developed flare-ups of hepatitis were successful in achieving a virological and biochemical response by addition of adefovir to lamivudine treatment. These data suggest that it is possible to continue lamivudine treatment even after emergence of YMDD mutants, at least until the patients develop a flare-up of hepatitis.

COMMENTS

Background

Although there is much evidence of the effectiveness of lamivudine in chronic hepatitis B patients, the number of patients with tyrosine-methionine-asparatate-asparatate (YMDD) motif mutation, which is linked to virological breakthrough, sometimes followed by a flare-up of hepatitis, is higher with prolonged lamivudine treatment. There is little information about the association between YMDD mutants and deterioration of liver function during long-term lamivudine treatment.

Research frontiers

The detection of YMDD mutants has mainly been by methods such as direct DNA sequencing, polymerase chain reaction (PCR)-restriction fragment length polymorphism, or reverse hybridization line probe assay, but these methods are labor-intensive, time-consuming, and have the risk of contamination. In this study, the authors demonstrated that fluorometric real-time PCR with the LightCycler probe hybridization assay was an easy, rapid and accurate method for the detection of YMDD mutants.

Innovations and breakthroughs

Regardless of hepatitis B e antigen positivity, the present study showed, by use of the LightCycler probe hybridization assay, that about half of the patients with YMDD mutants did not encounter virological breakthrough during long-term lamivudine treatment. Furthermore, all patients who developed flare-ups of hepatitis had a biochemical and virological response after adefovir was added to the lamivudine treatment.

Applications

The results suggest that it is possible to continue lamivudine treatment, even after the emergence of YMDD mutants, up to the time that the patients develop flare-ups of hepatitis.

Peer review

In this study, Murata *et al* retrospectively analyzed 61 HBV patients for up to 90 mo to find out the association between lamivudine resistance from its emergence with the hepatic deterioration. The positive finding of this study is the indication of continuation of lamivudine therapy in patients of genotype C having YMDD mutation until the stage of hepatic flare-up.The potential of Light Cycler probe hybridization for detection and monitoring of such mutants has also been elucidated. This is an important issue because lamivudine resistance is associated with progressive liver disease.

REFERENCES

1 **Liaw YF**, Chu CM. Hepatitis B virus infection. *Lancet* 2009;

373: 582-592
2 **Lavanchy D**. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat* 2004; **11**: 97-107
3 **Kashiwagi S**, Hayashi J, Ikematsu H, Nomura H, Kusaba T, Shingu T, Hayashida K, Kaji M. An epidemiologic study of hepatitis B virus in Okinawa and Kyushu, Japan. *Am J Epidemiol* 1983; **118**: 787-794
4 **Furusyo N**, Hayashi J, Sawayama Y, Kawakami Y, Kishihara Y, Kashiwagi S. The elimination of hepatitis B virus infection: changing seroepidemiology of hepatitis A and B virus infection in Okinawa, Japan over a 26-year period. *Am J Trop Med Hyg* 1998; **59**: 693-698
5 **Liaw YF**. Hepatitis B virus replication and liver disease progression: the impact of antiviral therapy. *Antivir Ther* 2006; **11**: 669-679
6 **Sumi H**, Yokosuka O, Seki N, Arai M, Imazeki F, Kurihara T, Kanda T, Fukai K, Kato M, Saisho H. Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. *Hepatology* 2003; **37**: 19-26
7 **Yuen MF**, Fung SK, Tanaka Y, Kato T, Mizokami M, Yuen JC, Wong DK, Yuan HJ, Sum SM, Chan AO, Wong BC, Lai CL. Longitudinal study of hepatitis activity and viral replication before and after HBeAg seroconversion in chronic hepatitis B patients infected with genotypes B and C. *J Clin Microbiol* 2004; **42**: 5036-5040
8 **Furusyo N**, Nakashima H, Kashiwagi K, Kubo N, Hayashida K, Usuda S, Mishiro S, Kashiwagi S, Hayashi J. Clinical outcomes of hepatitis B virus (HBV) genotypes B and C in Japanese patients with chronic HBV infection. *Am J Trop Med Hyg* 2002; **67**: 151-157
9 **Nakashima H**, Furusyo N, Kubo N, Kashiwagi K, Etoh Y, Kashiwagi S, Hayashi J. Double point mutation in the core promoter region of hepatitis B virus (HBV) genotype C may be related to liver deterioration in patients with chronic HBV infection. *J Gastroenterol Hepatol* 2004; **19**: 541-550
10 **Akuta N**, Kumada H. Influence of hepatitis B virus genotypes on the response to antiviral therapies. *J Antimicrob Chemother* 2005; **55**: 139-142
11 **Dienstag JL**, Goldin RD, Heathcote EJ, Hann HW, Woessner M, Stephenson SL, Gardner S, Gray DF, Schiff ER. Histological outcome during long-term lamivudine therapy. *Gastroenterology* 2003; **124**: 105-117
12 **Hadziyannis SJ**, Tassopoulos NC, Heathcote EJ, Chang TT, Kitis G, Rizzetto M, Marcellin P, Lim SG, Goodman Z, Ma J, Brosgart CL, Borroto-Esoda K, Arterburn S, Chuck SL. Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B for up to 5 years. *Gastroenterology* 2006; **131**: 1743-1751
13 **Gish RG**, Lok AS, Chang TT, de Man RA, Gadano A, Sollano J, Han KH, Chao YC, Lee SD, Harris M, Yang J, Colonno R, Brett-Smith H. Entecavir therapy for up to 96 weeks in patients with HBeAg-positive chronic hepatitis B. *Gastroenterology* 2007; **133**: 1437-1444
14 **Lai CL**, Gane E, Liaw YF, Hsu CW, Thongsawat S, Wang Y, Chen Y, Heathcote EJ, Rasenack J, Bzowej N, Naoumov NV, Di Bisceglie AM, Zeuzem S, Moon YM, Goodman Z, Chao G, Constance BF, Brown NA. Telbivudine versus lamivudine in patients with chronic hepatitis B. *N Engl J Med* 2007; **357**: 2576-2588
15 **Liaw YF**, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, Tanwandee T, Tao QM, Shue K, Keene ON, Dixon JS, Gray DF, Sabbat J. Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004; **351**: 1521-1531
16 **Papatheodoridis GV**, Dimou E, Dimakopoulos K, Manolakopoulos S, Rapti I, Kitis G, Tzourmakliotis D, Manesis E, Hadziyannis SJ. Outcome of hepatitis B e antigen-negative chronic hepatitis B on long-term nucleos(t)ide analog therapy starting with lamivudine. *Hepatology* 2005; **42**: 121-129
17 **Furusyo N**, Takeoka H, Toyoda K, Murata M, Tanabe Y, Kajiwara E, Shimono J, Masumoto A, Maruyama T, Nomura H, Nakamuta M, Takahashi K, Shimoda S, Azuma K, Sakai H, Hayashi J. Long-term lamivudine treatment for chronic hepatitis B in Japanese patients: a project of Kyushu University Liver Disease Study. *World J Gastroenterol* 2006; **12**: 561-567
18 **Allen MI**, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrrell DL, Brown N, Condreay LD. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. *Hepatology* 1998; **27**: 1670-1677
19 **Lok AS**, Zoulim F, Locarnini S, Bartholomeusz A, Ghany MG, Pawlotsky JM, Liaw YF, Mizokami M, Kuiken C. Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. *Hepatology* 2007; **46**: 254-265
20 **Zoulim F**, Locarnini S. Hepatitis B virus resistance to nucleos(t)ide analogues. *Gastroenterology* 2009; **137**: 1593-1608. e1-2
21 **Poordad F**, Chee GM. Viral resistance in hepatitis B: prevalence and management. *Curr Gastroenterol Rep* 2010; **12**: 62-69
22 **Whalley SA**, Brown D, Teo CG, Dusheiko GM, Saunders NA. Monitoring the emergence of hepatitis B virus polymerase gene variants during lamivudine therapy using the LightCycler. *J Clin Microbiol* 2001; **39**: 1456-1459
23 **Umeoka F**, Iwasaki Y, Matsumura M, Takaki A, Kobashi H, Tatsukawa M, Shiraha H, Fujioka S, Sakaguchi K, Shiratori Y. Early detection and quantification of lamivudine-resistant hepatitis B virus mutants by fluorescent biprobe hybridization assay in lamivudine-treated patients. *J Gastroenterol* 2006; **41**: 693-701
24 **Osiowy C**, Giles E. Evaluation of the INNO-LiPA HBV genotyping assay for determination of hepatitis B virus genotype. *J Clin Microbiol* 2003; **41**: 5473-5477
25 **Lai CL**, Dienstag J, Schiff E, Leung NW, Atkins M, Hunt C, Brown N, Woessner M, Boehme R, Condreay L. Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. *Clin Infect Dis* 2003; **36**: 687-696
26 **Lee CZ**, Lee HS, Huang GT, Yang PM, Sheu JC. Detection of YMDD mutation using mutant-specific primers in chronic hepatitis B patients before and after lamivudine treatment. *World J Gastroenterol* 2006; **12**: 5301-5305
27 **Matsuda M**, Suzuki F, Suzuki Y, Tsubota A, Akuta N, Hosaka T, Someya T, Kobayashi M, Saitoh S, Arase Y, Satoh J, Kobayashi M, Ikeda K, Miyakawa Y, Kumada H. YMDD mutants in patients with chronic hepatitis B before treatment are not selected by lamivudine. *J Med Virol* 2004; **74**: 361-366
28 **Kirishima T**, Okanoue T, Daimon Y, Itoh Y, Nakamura H, Morita A, Toyama T, Minami M. Detection of YMDD mutant using a novel sensitive method in chronic liver disease type B patients before and during lamivudine treatment. *J Hepatol* 2002; **37**: 259-265
29 **Lee CH**, Kim SO, Byun KS, Moon MS, Kim EO, Yeon JE, Yoo W, Hong SP. Predominance of hepatitis B virus YMDD mutants is prognostic of viral DNA breakthrough. *Gastroenterology* 2006; **130**: 1144-1152
30 **Hashimoto Y**, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, Hosaka T, Akuta N, Kobayashi M, Saito S, Suzuki Y, Kobayashi M, Arase Y, Ikeda K, Kumada H. Clinical and virological effects of long-term (over 5 years) lamivudine therapy. *J Med Virol* 2010; **82**: 684-691
31 **Sun J**, Wang Z, Ma S, Zeng G, Zhou Z, Luo K, Hou J. Clinical and virological characteristics of lamivudine resistance in chronic hepatitis B patients: a single center experience. *J Med Virol* 2005; **75**: 391-398
32 **Liaw YF**, Chien RN, Yeh CT. No benefit to continue lamivudine therapy after emergence of YMDD mutations. *Antivir Ther* 2004; **9**: 257-262
33 **Yuen MF**, Seto WK, Chow DH, Tsui K, Wong DK, Ngai VW, Wong BC, Fung J, Yuen JC, Lai CL. Long-term lamivudine therapy reduces the risk of long-term complications of chron-

ic hepatitis B infection even in patients without advanced disease. *Antivir Ther* 2007; **12**: 1295-1303

34 **Pallier C**, Castéra L, Soulier A, Hézode C, Nordmann P, Dhumeaux D, Pawlotsky JM. Dynamics of hepatitis B virus resistance to lamivudine. *J Virol* 2006; **80**: 643-653

35 **Ono SK**, Kato N, Shiratori Y, Kato J, Goto T, Schinazi RF, Carrilho FJ, Omata M. The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. *J Clin Invest* 2001; **107**: 449-455

36 **Lampertico P**, Viganò M, Manenti E, Iavarone M, Lunghi G, Colombo M. Adefovir rapidly suppresses hepatitis B in HBeAg-negative patients developing genotypic resistance to lamivudine. *Hepatology* 2005; **42**: 1414-1419

37 **Rapti I**, Dimou E, Mitsoula P, Hadziyannis SJ. Adding-on versus switching-to adefovir therapy in lamivudine-resistant HBeAg-negative chronic hepatitis B. *Hepatology* 2007; **45**: 307-313

38 **Lok AS**, McMahon BJ. Chronic hepatitis B: update 2009. *Hepatology* 2009; **50**: 661-662

39 **Keeffe EB**, Dieterich DT, Han SH, Jacobson IM, Martin P, Schiff ER, Tobias H. A treatment algorithm for the management of chronic hepatitis B virus infection in the United States: 2008 update. *Clin Gastroenterol Hepatol* 2008; **6**: 1315-1341; quiz 1286

40 **Lampertico P**, Viganò M, Manenti E, Iavarone M, Sablon E, Colombo M. Low resistance to adefovir combined with lamivudine: a 3-year study of 145 lamivudine-resistant hepatitis B patients. *Gastroenterology* 2007; **133**: 1445-1451

S- Editor Sun H **L- Editor** Cant MR **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2953

World J Gastroenterol 2011 June 28; 17(24): 2953-2957
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2011 Baishideng. All rights reserved.

BRIEF ARTICLE

Total embolization of the main splenic artery as a supplemental treatment modality for hypersplenism

Xin-Hong He, Wen-Tao Li, Wei-Jun Peng, Guo-Dong Li, Sheng-Ping Wang, Li-Chao Xu

Xin-Hong He, Wen-Tao Li, Wei-Jun Peng, Guo-Dong Li, Sheng-Ping Wang, Li-Chao Xu, Department of Radiology, Fudan University Shanghai Cancer Center, Shanghai 200032, China
Xin-Hong He, Wen-Tao Li, Wei-Jun Peng, Guo-Dong Li, Sheng-Ping Wang, Li-Chao Xu, Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China
Author contributions: He XH, Li WT and Peng WJ designed the research; He XH, Li WT, Li GD, Wang SP and Xu LC performed the research; Li WT, Peng WJ and Li GD provided the new reagents/analytic tools; Wang SP and Xu LC analyzed the data; He XH and Wang SP wrote the paper.
Correspondence to: Wen-Tao Li, Professor, Department of Radiology, Fudan University Shanghai Cancer Center, Shanghai 200032, China. wentao.li.sh@gmail.com
Telephone: +86-21-64175590 **Fax:** +86-21-64049870
Received: October 7, 2010 **Revised:** December 5, 2010
Accepted: December 12, 2010
Published online: June 28, 2011

Abstract

AIM: To study the safety and feasibility of total embolization of the main splenic artery as a supplemental treatment modality for hypersplenism with thrombocytopenia or leukocytopenia accompanying liver cirrhosis.

METHODS: Fifteen consecutive patients with hypersplenism due to cirrhosis were enrolled in this study from January 2006 to June 2010. All patients underwent total embolization of the main splenic artery. Clinical symptoms, white blood cell (WBC) and platelet (PLT) counts, splenic volume, and complications of the patients were recorded. The patients were followed up for 1 and 6 mo, and 1, 2, 3 years, respectively, after operation.

RESULTS: Total embolization of the main splenic artery was technically successful in all patients. Minor complications occurred in 13 patients after the procedure, but no major complications were found. The WBC and PLT counts were significantly higher and the residual splenic volume was significantly lower 1 and 6 mo, and 1, 2, 3 years after the procedure than before the procedure ($P < 0.01$). Moreover, the residual splenic volume increased very slowly with the time after embolization. All patients were alive during the follow-up period.

CONCLUSION: Total embolization of the main splenic artery is a safe and feasible procedure and may serve as a supplemental treatment modality for hypersplenism with thrombocytopenia or leukocytopenia accompanying liver cirrhosis.

© 2011 Baishideng. All rights reserved.

Key words: Liver cirrhosis; Hypersplenism; Coil embolization; Splenic artery

Peer reviewer: Yasushi Matsuzaki, Associated Professor, Division of Gastroenterology and Hepatology, Graduate School of Comprehensive Human Sciences and University Hospital, 1-1-1, Tennodai, Tsukuba 305-8575, Japan

He XH, Li WT, Peng WJ, Li GD, Wang SP, Xu LC. Total embolization of the main splenic artery as a supplemental treatment modality for hypersplenism. *World J Gastroenterol* 2011; 17(24): 2953-2957 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2953.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2953

INTRODUCTION

Partial splenic embolization (PSE) is a non-surgical procedure for hypersplenism resulting from hepatic disease and can thus avoid the disadvantages of splenectomy[1]. It has been shown that PSE can increase peripheral blood cell counts[2-5]. However, PSE often results in a number of complications, including daily intermittent fever, abdominal pain, nausea, vomiting, postemboliza-

tion syndrome, splenic abscess and rupture, pneumonia, refractory ascites, pleural effusion, and gastrointestinal bleeding[2-8].

Total embolization of the splenic artery is a safe and effective procedure for splenic artery aneurysms[9-13]. Moreover, stainless steel spring coils are used to embolize the main branch of splenic artery to increase the platelet (PLT) count before splenectomy[14]. To date, no report is available on the treatment of hypersplenism with total embolization of the main splenic artery. The present study was to study the safety and feasibility of total embolization of the main splenic artery for hypersplenism with thrombocytopenia or leukocytopenia accompanying liver cirrhosis.

MATERIALS AND METHODS

Patients

Fifteen consecutive patients (10 males and 5 females with a mean age of 50.07 ± 8.98 years, ranging 38-60 years) with hypersplenism due to cirrhosis were enrolled in this study from January 2006 to June 2010 and subsequently underwent computed tomography (CT) follow-up at our hospital. The causes of cirrhosis were hepatitis B virus (HBV) infection in 13 patients and hepatitis C virus (HCV) infection in 2 patients. The patients were diagnosed as hypersplenism based on their history, clinical laboratory tests, ultrasonography and CT. The protocol was approved by The Ethics Committee of Fudan University and the patients provided their written informed consent.

The inclusion criteria were those with hypersplenism, HBV/HCV-related active cirrhosis, neutropenia (neutrophil count $\leqslant 2.0 \times 10^9$ cells/L), thrombocytopenia (PLT count $\leqslant 50 \times 10^9$ cells/L) or both, and follow-up time > 2 years. Those with severe jaundice [serum total bilirubin (TB) level $\geqslant$ 81.4 μmol/L] or spontaneous bacterial peritonitis were excluded from the study.

Hypersplenism was classified as Child-Pugh class A in 10 patients, class B in 3 patients, and class C in 2 patients. The demographics of these patients are summarized in Table 1.

Endovascular techniques

Metallic coils and gelfoam were used as embolization materials, either alone or in combination. In general, the embolization coils used in this series were standard 0.089-cm (0.035-in.) fibered coils, microcoils (Tornado; Cook Inc., Bloomington, IN, USA).

Embolization was performed in all patients *via* the femoral artery. Selective angiography of the celiac trunk, splenic artery, and superior mesenteric artery was performed *via* the right femoral artery with a 5-Fr diagnostic catheter (Cook). Patency of the collateral arteries connected to the hilar splenic artery from the left gastric artery or from the gastroepiploic artery on a celiac arteriogram was monitored to avoid total splenic infarction. Total embolization of the main splenic artery was performed after confirmation of these connections.

All embolization procedures were performed by 2 experienced interventional radiologists. Details of the coiling procedures have been described previously[9,11,15]. Selective splenic artery, celiac, and superior mesenteric artery angiograms were performed to confirm occlusion of the main splenic artery and patency of the collateral arteries after embolization. Preoperative antibiotic prophylaxis was used routinely for 3 d. Following embolization, patients were monitored and antibiotics were continued after the procedure for several days to avoid infectious complications.

Follow-up protocol and postoperative outcome evaluation

All patients were followed up at our outpatient clinic. Peripheral blood cell parameters, including white blood cell (WBC), PLT, and red blood cell counts, were monitored at different time points prior to the procedure and on days 3, 14, and 30 after the procedure, then at 6-mo intervals during the 3-year follow-up period. To determine the effect of embolization on liver function, serum levels of aspartate aminotransferase, alanine aminotransferase, TB, and albumin were measured and prothrombin time was calculated at the same follow-up time points as above before and after the procedure. The procedure-related frequency and type of complications were recorded.

Abdominal CT scans were routinely performed before and 2 wk after the procedure, and then every 6 mo during the follow-up. Based on enhanced CT images, the pretreatment splenic volume and the post-embolization residual splenic volume were measured and compared on a workstation (Siemens Syngo MMWP VEZIA) using the volumetric analysis software. The infracted splenic volume (mL) was measured by subtracting the noninfarcted splenic volume from the pretreatment splenic volume. The splenic infarction rate was calculated by dividing the infarcted splenic volume by the pretreatment splenic volume (× 100%).

The procedure-related complications were divided into major and minor ones. Major complications associated with the procedure, including splenic abscess, splenic rupture, pneumonia, refractory ascites or pleural effusion, gastrointestinal bleeding, rupture of varices, and hepatic failure, were defined as complicated disease requiring surgical intervention or prolonged postoperative hospital stay time of more than 30 d. Minor complications, including abdominal pain, fever, vomiting, abdominal fullness, and appetite loss, were defined as those that lead to no consequential events and can be tolerated by the patients.

Statistical analysis

All data were expressed as mean ± SD. Changes in WBC and PLT counts after PSE were evaluated by paired t test. The variables between the 2 groups were compared by Mann-Whitney test, χ^2-test or Fisher's exact test when ap-

Table 1 Outcomes of total embolization of the main splenic artery in 15 patients

Pa./age (yr)/sex	Virus	Child-Pugh	WBC count (× 10^9/L)						Platelets (× 10^9/L)						Splenic volumn (cm^3)						Compli-cation	Follow-up (mo)	Out-comes
			Pre-EM	1 mo	6 mo	1 yr	2 yr	3 yr	Pre-EM	1 mo	6 mo	1 yr	2 yr	3 yr	Pre-EM	1 mo	6 mo	1 yr	2 yr	3 yr			
1/48/F	B	A	1.3	7.8	6.5	5.3	4.9	5.1	35	181	142	136	132	128	829	367	265	265	312	312	AP, F, V	52	Alive
2/52/M	B	B	1.8	9.8	5.3	5.6	5.6	4.4	24	191	156	148	145	139	768	258	326	326	326	367	F, V	48	Alive
3/60/M	B	A	1.6	8.6	6.5	6.3	4.2	6.4	33	233	185	157	132	142	869	369	328	328	328	305	AP, F	47	Alive
4/51/M	B	A	1.8	8.3	5.4	7.2	6.2	4.6	45	173	158	156	148	132	815	289	289	287	250	285	F, V	45	Alive
5/34/F	B	A	1.1	7.8	7.2	6.8	5.2	4.5	48	165	161	152	153	148	724	366	242	235	235	235	AP, V	41	Alive
6/49/M	B	C	1.4	8.6	4.8	4.6	4.4	4.8	43	144	43	139	132	123	698	278	278	278	278	278	AP, V	40	Alive
7/50/F	B	A	1.5	8.5	5.8	4.8	4.6	4.2	25	156	145	146	134	129	758	325	325	312	314	314		37	Alive
8/59/M	C	B	1.1	7.8	6.3	6.7	7.3	4.8	36	143	145	132	128	132	846	319	319	310	310	310	AP, F, V	36	Alive
9/28/M	B	A	1.5	9.6	6.0	5.1	5.2	5.3	48	213	167	164	156	147	687	247	247	247	249	276	AP, F,	36	Alive
10/55/M	B	A	0.8	7.3	6.9	5.6	5.4	4.5	32	121	125	131	128	124	784	305	305	298	320	320	AP, F, V	42	Alive
11/58/F	B	C	1.7	6.8	8.5	6.8	3.9	4.2	26	163	184	146	139	132	755	362	362	362	362	345	AP, F	38	Alive
12/46/M	B	A	0.9	5.3	4.8	3.8	4.5		48	146	135	141	136		732	247	285	285	285		AP, F, V	28	Alive
13/58/F	C	A	1.2	8.2	3.8	4.8	5.0		32	153	138	146	134		848	328	328	318	315		AP, F	30	Alive
14/55/M	B	B	1.4	8.3	5.2	4.5	4.4		35	164	176	163	165		683	361	356	346	327			26	Alive
15/48/M	B	A	1.6	8.8	6.3	6.2	6.4		38	241	220	168	137		752	298	305	315	315		AP, F	24	Alive

Pre-EM: Pre-embolization; AP: Abdominal pain; F: Fever; V: Vomiting; WBC: White blood cell.

propriate. All statistical analyses were performed using the SPSS package, version 13.0 (SPSS, Chicago, Illinois, USA).

RESULTS

Primary procedure results

Total embolization of the main splenic artery was technically successful in all patients, with no procedure-related complications. The mean postoperative hospital stay time was 8.40 ± 2.53 d (range, 5-15 d) after the procedure and the 30 d mortality rate was zero.

Minor complications occurred in 13 patients with no major complications found after the procedure. The most frequent side effects were abdominal pain, fever, and nausea. Prolonged fever, lasting over 15 d after the procedure, developed in 1 case. These side effects were controlled after conservative therapy.

Changes in peripheral blood cell counts after embolization

The outcomes of total embolization of the main splenic artery in 15 patients are shown in Table 1. All patients were assessed 1 and 6 mo, and 1, 2, 3 years after the procedure. The patients were followed up for 38.0 ± 8.32 mo (range, 24-52 mo). The mean WBC count increased from 1.4 (0.3) × 10^9/L before the procedure to 8.1 (1.1) × 10^9/L, 6.0 (1.2) × 10^9/L, 5.6 (1.0) × 10^9/L, 5.1 (0.9) × 10^9/L, and 4.8 (0.9) × 10^9/L, respectively, 1 and 6 mo, and 1, 2, 3 years after the procedure ($P < 0.01$).

The mean PLT count increased from 36.5 (8.3) × 10^9/L before the procedure to 172 (34.1) × 10^9/L, 152 (38.7) × 10^9/L, 148 (11.6) × 10^9/L, 140 (11.1) × 10^9/L, and 134 (8.6) × 10^9/L, respectively, 1 and 6 mo, and 1, 2, 3 years after the procedure ($P < 0.01$).

Changes in splenic volume after embolization

The mean splenic volume decreased from 769.87 (60.51) cm^3 before the procedure to 314.60 (44.52) cm^3, 304.0 (36.10) cm^3, 300.80 (35.20) cm^3, 301.73 (35.17) cm^3, and 306.00 (32.02) cm^3, respectively, 1 and 6 mo, and 1, 2, 3 years after the procedure ($P < 0.05$). During the follow-up, the residual splenic volume in these patients increased a very slowly. The mean infarction rate of the spleen was 60% (range, 59%-61%) 3 years after the procedure. No death occurred during the follow-up.

DISCUSSION

The results of his study show that total embolization of the main splenic artery with coils is a safe and feasible procedure for hypersplenism due to liver cirrhosis. The peripheral blood cell parameters including WBC and PLT counts increased significantly during the follow-up and the residual splenic volume increased very slowly after embolization.

Hypersplenism is a well-known complication of portal hypertension due to cirrhosis, which can result in thrombocytopenia and/or leukocytopenia. Splenectomy can eliminate hypersplenism-induced blood cell destruction, but the incidence of severe complications after splenectomy is 9.6%-26.6% whether laparoscopy or open splenectomy is performed[16-18]. In addition, splenectomy is often associated with an increased long-term risk of septic events[16-18].

Although PSE is an effective alternative to splenectomy to increase the peripheral blood cell counts[1-5], severe complications of PSE, such as splenic abscess, splenic rupture, pneumonia, refractory ascites or pleural effusion, and gastrointestinal bleeding[6-8], greatly limit its use. Furthermore, the complications of PSE are correlated with the infracted splenic volume. In addition, when 50% or less than 50% of the spleen is embolized, hypersplenism would relapse shortly after PSE[2,8]. Therefore, to ensure a sustained increase in PLT and leukocyte counts,

the splenic infarction rate should be greater than 50%[8], which, however, inevitably results in severe complications. To increase the PLT and leukocyte counts and reduce the rate of severe complications, total embolization of the main splenic artery was performed for hypersplenism due to liver cirrhosis in the present study.

The key procedure for reducing the severe complications and ensuring the sustained increase in PLT and leukocyte counts is to confirm the patency of collateral arteries connected to the hilar splenic artery from the left gastric artery or from the gastroepiploic artery. If these connections are absent or incomplete, total embolization of the main splenic artery should not be performed because the procedure may result in more severe complications.

When the main splenic artery is completely embolized, the main blood flow supplying the spleen is stopped, but the collateral arteries connected to the hilar splenic artery from the left gastric artery or from the gastroepiploic artery may provide a small amount of blood for the spleen to avoid complete infarction of the spleen. Thus, most of the spleen should be embolized with reservation of a partial normal spleen. Thus, the PLT and leukocyte counts increase after the procedure, and the occurrence of severe complications can be circumvented. In this study, the safety and feasibility of total embolization for hypersplenism of the main splenic artery were studied.

As compared with PSE, total embolization of the main splenic artery has the following advantages, including a low risk of procedure-related complications, persistent maintenance of normal WBC and PLT counts, and a very slow increase in residual splenic volume.

Although these results are encouraging, this study had the following limitations. First, it was not a comparative study and the number of patients was small with no control group. Future randomized multicenter trials comparing PSE with total embolization are needed to determine their long-term clinical efficacy and risk of complications. Second, total embolization could not be performed in patients with no or incomplete collateral arteries.

In conclusion, total embolization of the main splenic artery is a safe and feasible procedure for hypersplenism with thrombocytopenia or leukocytopenia accompanying liver cirrhosis and may serve as a supplemental treatment modality for it. Further clinical trials and expanded follow-up studies are needed to confirm its safety.

COMMENTS

Background

Partial splenic embolization (PSE) is a non-surgical procedure for hypersplenism resulting from hepatic disease, thus avoiding the disadvantages of splenectomy. However, after PSE, patients often experience complications, including daily intermittent fever, abdominal pain, nausea, vomiting, and postembolization syndrome.

Research frontiers

Total embolization of the splenic artery has been widely used in treatment of splenic artery aneurysms, but no report is available on treatment of hypersplenism with it. In this study, total embolization of the main splenic artery for hypersplenism with thrombocytopenia or leukocytopenia accompanying liver cirrhosis was studied.

Innovations and breakthroughs

Total embolization of the main splenic artery was devised for the treatment of hypersplenism with thrombocytopenia or leukocytopenia accompanying liver cirrhosis. All procedures were performed under fluoroscopic control. This is the first study reporting the treatment of hypersplenism with total embolization of the main splenic artery.

Applications

Total embolization of the main splenic artery is a safe and feasible procedure and may serve as a supplemental treatment modality for hypersplenism with thrombocytopenia or leukocytopenia accompanying liver cirrhosis with a low complication rate and a good mid-term clinical efficacy.

Terminology

Hypersplenism is a well-known complication of portal hypertension due to cirrhosis, which can result in thrombocytopenia and leukocytopenia.

Peer review

The finding in this study is interesting. Further study is needed confirm its safety in a much larger series of patients.

REFERENCES

1 **Yoshida H**, Mamada Y, Taniai N, Tajiri T. Partial splenic embolization. *Hepatol Res* 2008; **38**: 225-233

2 **Sangro B**, Bilbao I, Herrero I, Corella C, Longo J, Beloqui O, Ruiz J, Zozaya JM, Quiroga J, Prieto J. Partial splenic embolization for the treatment of hypersplenism in cirrhosis. *Hepatology* 1993; **18**: 309-314

3 **N'Kontchou G**, Seror O, Bourcier V, Mohand D, Ajavon Y, Castera L, Grando-Lemaire V, Ganne-Carrie N, Sellier N, Trinchet JC, Beaugrand M. Partial splenic embolization in patients with cirrhosis: efficacy, tolerance and long-term outcome in 32 patients. *Eur J Gastroenterol Hepatol* 2005; **17**: 179-184

4 **Tajiri T**, Onda M, Yoshida H, Mamada Y, Taniai N, Kumazaki T. Long-term hematological and biochemical effects of partial splenic embolization in hepatic cirrhosis. *Hepatogastroenterology* 2002; **49**: 1445-1448

5 **Miyake Y**, Ando M, Kaji E, Toyokawa T, Nakatsu M, Hirohata M. Partial splenic embolization prior to combination therapy of interferon and ribavirin in chronic hepatitis C patients with thrombocytopenia. *Hepatol Res* 2008; **38**: 980-986

6 **Sakai T**, Shiraki K, Inoue H, Sugimoto K, Ohmori S, Murata K, Takase K, Nakano T. Complications of partial splenic embolization in cirrhotic patients. *Dig Dis Sci* 2002; **47**: 388-391

7 **Hayashi H**, Beppu T, Okabe K, Masuda T, Okabe H, Baba H. Risk factors for complications after partial splenic embolization for liver cirrhosis. *Br J Surg* 2008; **95**: 744-750

8 **Zhu K**, Meng X, Qian J, Huang M, Li Z, Guan S, Jiang Z, Shan H. Partial splenic embolization for hypersplenism in cirrhosis: a long-term outcome in 62 patients. *Dig Liver Dis* 2009; **41**: 411-416

9 **Loffroy R**, Guiu B, Cercueil JP, Lepage C, Cheynel N, Steinmetz E, Ricolfi F, Krausé D. Transcatheter arterial embolization of splenic artery aneurysms and pseudoaneurysms: short- and long-term results. *Ann Vasc Surg* 2008; **22**: 618-626

10 **Venkatesh SK**, Kumar S, Baijal SS, Phadke RV, Kathuria MK, Gujral RB. Endovascular management of pseudoaneurysms of the splenic artery: experience with six patients. *Australas Radiol* 2005; **49**: 283-288

11 **Laganà D**, Carrafiello G, Mangini M, Fontana F, Dizonno M, Castelli P, Fugazzola C. Endovascular treatment of splenic artery aneurysms. *Radiol Med* 2005; **110**: 77-87

12 **Piffaretti G**, Tozzi M, Lomazzi C, Rivolta N, Riva F, Caronno R, Castelli P. Splenic artery aneurysms: postembolization syndrome and surgical complications. *Am J Surg* 2007; **193**: 166-170

13 **Laganà D**, Carrafiello G, Mangini M, Dionigi G, Caronno R, Castelli P, Fugazzola C. Multimodal approach to endovascu-

lar treatment of visceral artery aneurysms and pseudoaneurysms. *Eur J Radiol* 2006; **59**: 104-111

14 **Takahashi T**, Arima Y, Yokomuro S, Yoshida H, Mamada Y, Taniai N, Kawano Y, Mizuguchi Y, Shimizu T, Akimaru K, Tajiri T. Splenic artery embolization before laparoscopic splenectomy in children. *Surg Endosc* 2005; **19**: 1345-1348

15 **Guillon R**, Garcier JM, Abergel A, Mofid R, Garcia V, Chahid T, Ravel A, Pezet D, Boyer L. Management of splenic artery aneurysms and false aneurysms with endovascular treatment in 12 patients. *Cardiovasc Intervent Radiol* 2003; **26**: 256-260

16 **Winslow ER**, Brunt LM. Perioperative outcomes of laparoscopic versus open splenectomy: a meta-analysis with an emphasis on complications. *Surgery* 2003; **134**: 647-653; discussion 654-655

17 **Kojouri K**, Vesely SK, Terrell DR, George JN. Splenectomy for adult patients with idiopathic thrombocytopenic purpura: a systematic review to assess long-term platelet count responses, prediction of response, and surgical complications. *Blood* 2004; **104**: 2623-2634

18 **Watanabe Y**, Horiuchi A, Yoshida M, Yamamoto Y, Sugishita H, Kumagi T, Hiasa Y, Kawachi K. Significance of laparoscopic splenectomy in patients with hypersplenism. *World J Surg* 2007; **31**: 549-555

S- Editor Tian L **L- Editor** Wang XL **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2958

World J Gastroenterol 2011 June 28; 17(24): 2958-2964
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2011 Baishideng. All rights reserved.

BRIEF ARTICLE

Antitumor activity of mutant bacterial cytosine deaminase gene for colon cancer

Long-Ying Deng, Jian-Ping Wang, Zhi-Fu Gui, Li-Zong Shen

Long-Ying Deng, Jian-Ping Wang, Zhi-Fu Gui, Li-Zong Shen, Division of Gastrointestinal Surgery, Department of General Surgery, First Affiliated Hospital, Nanjing Medical University, 300 Guangzhou, Nanjing 210029, Jiangsu Province, China
Author contributions: Shen LZ designed the research; Deng LY, Wang JP and Gui ZF performed the research and analyzed the data; Deng LY and Shen LZ wrote the paper; Deng LY and Wang JP contributed equally to this work.
Supported by The Social Development Foundation from Science and Technology Bureau of Nanjing, No. 200605010
Correspondence to: Li-Zong Shen, PhD, MD, Division of Gastrointestinal Surgery, Department of General Surgery, First Affiliated Hospital, Nanjing Medical University, 300 Guangzhou, Nanjing 210029, Jiangsu Province, China. shenlz@163.com
Telephone: +86-25-85038020 **Fax:** +86-25-85038020
Received: November 17, 2010 **Revised:** March 11, 2011
Accepted: March 18, 2011
Published online: June 28, 2011

Abstract

AIM: To evaluate bacterial cytosine deaminase (bCD) mutant D314A and 5-fluorocytosine (5-FC) for treatment of colon cancer in a mouse model.

METHODS: Recombinant lentivirus vectors that contained wild-type bCD gene (bCDwt), and bCD mutant D314A gene (bCD-D314A) with green fluorescence protein gene were constructed and used to infect human colon carcinoma LoVo cells, to generate stable transfected cells, LoVo/null, LoVo/bCDwt or LoVo/bCD-D314A. These were injected subcutaneously into Balb/c nude mice to establish xenograft models. Two weeks post-LoVo cell inoculation, PBS or 5-FC (500 mg/kg) was administered by intraperitoneal (i.p.) injection once daily for 14 d. On the day after LoVo cell injection, mice were monitored daily for tumor volume and survival.

RESULTS: Sequence analyses confirmed the construction of recombinant lentiviral plasmids that contained bCDwt or bCD-D314A. The lentiviral vector had high efficacy for gene delivery, and RT-PCR showed that bCDwt or bCD-D314A gene was transferred to LoVo cells. Among these treatment groups, gene delivery or 5-FC administration alone had no effect on tumor growth. However, bCDwt/5-FC or bCD-D314A/5-FC treatment inhibited tumor growth and prolonged survival of mice significantly (P < 0.05). Importantly, the tumor volume in the bCD-D314A/5-FC-treated group was lower than that in the bCDwt/5-FC group (P < 0.05), and bCD-D314A plus 5-FC significantly prolonged survival of mice in comparison with bCDwt plus 5-FC (P < 0.05).

CONCLUSION: The bCD mutant D314A enhanced significantly antitumor activity in human colon cancer xenograft models, which provides a promising approach for human colon carcinoma therapy.

© 2011 Baishideng. All rights reserved.

Key words: Suicide gene therapy; Bacterial cytosine deaminase; Mutant; D314A; 5-fluorocytosine; Colon cancer

Peer reviewer: Dr. Dinesh Vyas, Department of Minimally and Endoscopic Surgery, St John Mercy Hospital, 851 E Fifth Street, Washington, MO 63090, United States

Deng LY, Wang JP, Gui ZF, Shen LZ. Antitumor activity of mutant bacterial cytosine deaminase gene for colon cancer. *World J Gastroenterol* 2011; 17(24): 2958-2964 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2958.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2958

INTRODUCTION

Colorectal cancer has one of the highest mortalities worldwide[1]. Conventional therapies consist of surgery, chemotherapy, radiotherapy, and biotherapy. Despite advances in the treatment of colorectal cancer, the prognosis for locally advanced or metastatic disease remains relatively

poor[2]. Therefore, it is crucial to develop novel therapeutic strategies, not only to completely cure cancer, but also to prevent its recurrence. Among these approaches, gene-directed enzyme-prodrug therapy (GDEPT) has received considerable attention[3,4].

Suicide gene therapy for cancer is an appealing alternative to standard methods of chemotherapy because most chemotherapeutic agents lack tumor specificity. Classic chemotherapeutic agents are often unable to distinguish tumor cells from normal dividing cells, which results in indiscriminate toxic effects. In contrast, suicide gene therapy allows for specific targeting of the tumor while preventing damage to normal cells. This is accomplished by introducing a gene that encodes a prodrug-activating enzyme into cancer cells. Although delivery systems have not been fully optimized, delivery of the gene is typically done using either a viral vector (retrovirus or adenovirus) or by other non-viral means[5-7]. Once the gene is delivered into the cancer cell, a non-toxic prodrug is administered. The enzyme converts the non-toxic prodrug into its active and lethal form that results in cancer cell death. *Escherichia coli* or bacterial cytosine deaminase (bCD) is responsible for the activation of the non-toxic prodrug 5-fluorocytosine (5-FC) to its toxic form, 5-fluorouracil (5-FU)[8]. The absence of an endogenous cytosine deaminase in mammalian cells provides for deamination of 5-FC only in cells that express bCD. This is followed by the conversion of 5-FU into its deoxyribonucleoside, fluorodeoxyuridine (FUdR) by thymidine phosphorylase. Upon phosphorylation of FUdR by endogenous thymidine kinase, thymidylate synthase is irreversibly inhibited by the product, 5FdUMP, thereby preventing dTTP formation and ultimately leading to inhibition of DNA synthesis.

One of the key advantages of the bCD/5-FC enzyme/prodrug system is the phenomenon known as the "bystander effect", which is defined as the killing of untransfected cells neighboring those cells transfected with the suicide gene[8]. This type of killing has been described extensively with regard to the herpes simplex virus-1 thymidine kinase and ganciclovir enzyme/prodrug system, and occurs primarily by the transfer of toxic antimetabolites through gap junctions[9,10]. Unlike phosphorylated ganciclovir, 5-FU is a small, uncharged molecule that can pass freely in and out of the cell by diffusion. Consequently, cell-cell contact is not required for the bystander effect with bCD/5-FC; an advantage for those cell types with limited gap junctions[11]. bCD has been used successfully in gene therapy for a variety of animal tumor models and is currently under investigation for the treatment of human cancers[12-15].

The limiting factors for successful suicide GDEPT are transfection efficiency and the ability of the enzyme to turn over the prodrug, which is an analog of its natural substrate. From a kinetic perspective, 5-FC is a poor substrate for bCD (Km = 3.3 mmol/L) compared with its native substrate, cytosine (Km = 0.2 mmol/L)[16]. Thus, high doses of this prodrug must be administered to achieve cell killing. The plasma levels of 5-FC required to obtain a significant amount of active metabolites may lead to adverse effects. This is observed with 5-FC, whereas deamination by CD of bacterial intestinal microflora into 5-FU is responsible for side effects[17]. Other studies have suggested that the CD from *Saccharomyces cerevisiae* (yCD) displays a kinetic advantage towards 5-FC over bCD[18]. However, yCD is considerably less thermostable than bCD; a characteristic that may make the bacterial enzyme a preferable catalytic system for gene therapy.

Fortunately, Mahan *et al*[16,19] have used random mutagenesis to create novel bCDs that demonstrate an increased preference for 5-FC over cytosine. Among these mutants isolated, the mutant D314A [substitution of an alanine (A) for the aspartic acid (D) at position 314 of bCD] enzyme demonstrates a dramatic decrease in cytosine activity (17-fold), as well as a slight increase in activity toward 5-FC (twofold), which indicates that mutant D314A prefers the prodrug over cytosine by almost 20-fold. Despite the thermolability of yCD, others have suggested that yCD is superior to bCD in gene therapy settings because of a 23-fold relative substrate preference for 5-FC that is displayed by yCD[18]. However, given the thermostability of bCD and the 19-fold relative substrate preference that the bCD mutant D314A displays towards 5-FC, bCD D314A may be a superior suicide gene to yCD. These results indicate that bCD mutant D314A is a superior candidate for suicide gene therapy. Recently, this mutant D314A has been demonstrated to enhance cytotoxicity of human glioma and pancreatic cancer cells, and to increase therapeutic efficacy against human glioma and human pancreatic tumor xenografts, especially combined with radiotherapy[20,21]. However, there have been only a few studies of bCD mutants in colorectal cancer.

Previously, we have used the bCD gene to treat colon cancer, and have found that the efficacy of wild-type bCD is not sufficient to abolish cancer cells *in vitro* or *in vivo*, therefore, combination therapy with other genes, such as interleukin-2 or interferon-γ, is needed to improve the cytotoxicity of bCD[7,22,23]. Recently, we have constructed bCD-D314A using site-directed mutagenesis[19], and have demonstrated that it has significantly increased cytotoxicity in human colon cancer cell line LoVo, compared with wild-type bCD (bCDwt) *in vitro*[24].

The aim of the present study was to investigate whether bCD-D314A suicide gene and 5-FC prodrug therapy produce increased therapeutic efficacy *in vivo* for human colon cancer in nude mice using lentiviral vectors. The results presented here indicated that mutant bCD-D314A was able to significantly enhance antitumor efficacy in human colon cancer xenograft models *in vivo* compared with bCDwt.

MATERIALS AND METHODS

Cells and cell culture

Human colon cancer cell line LoVo (CCL-229; ATCC, Manassas, VA, USA) was cultured in RPMI-1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). HEK 293T (human embryonic kidney 293T cell line containing SV40 large T antigen) (CRL-11268; ATCC) was cultured in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% cosmic calf serum (Hy-

clone, Logan, UT, USA), 2 mmol/L L-glutamine (Sigma, St. Louis, MO, USA), 100 U/mL penicillin (Sigma) and 0.1 mg/mL streptomycin (Sigma). LoVo and HEK 293T cells were in a 5% CO_2-humidified atmosphere at 37℃.

Reagents, animals, plasmids and vectors

5-FC was obtained from Sigma. Restriction enzymes *Hin*dⅢ, *Kpn*Ⅰ, *Nhe*Ⅰ, *Eco*RⅠ and *Dpn*Ⅰ, T4 DNA ligase, pfu DNA polymerase, DNA marker DL2000 and PCR reagents were obtained from Takara (Otsu, Shiga, Japan). Primers were chemically synthesized by Shanghai Generay Co. Ltd. (Shanghai, China). The plasmid DNA extraction (Mini) kit was provided by Qiagen (Crawley, West Sussex, UK). TRIzol and Lipofectamine 2000™ transfection reagent were obtained from Invitrogen.

Female athymic Balb/c (nu/nu) nude mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China) and were housed under aseptic conditions in micro-isolator cages, which were approved by the local Institutional Animal Care and Use Committee.

The plasmid pcDNA3.1/bCDwt that contained whole-length wild-type bCD gene, and pcDNA3.1/bCD-D314A that contained the mutant *D314A* gene, were prepared and stored in our department. The pLJM1-GFP lentivirus vector with green fluorescence protein (GFP) gene was a generous gift from Prof. J Li (Nanjing Medical University).

Recombinant bCDwt or bCD-D314A plasmids construction and identification

The pcDNA3.1-bCDwt and pcDNA3.1-bCD-D314A plasmids were all double digested with *Hin*dⅢ and *Kpn*Ⅰ. The products of enzyme digestion were connected to the lentiviral vector pLJM1-GFP, which was double digested with *Nhe*Ⅰ and *Eco*RⅠ, to produce pLJM1-bCDwt-GFP and pLJM1-bCD-D314A-GFP. These plasmids were then transformed into *E. coli* XL1-Blue. The colonies were selected for PCR identification. The sense sequence of bCD primers was 5'-CGCAAATGGGCGGTAGGCGTG-3', whereas the antisense sequence was 5'AATTCTCAACGTTTGTAATCGATGG-3'. These recombinant plasmids were extracted and sent to BGI Sequencing Company (Shanghai, China) for sequencing.

Recombinant lentivirus construction, cell infection and stable cell line generation

To produce recombinant lentiviruses that encoded bCDwt, bCD-D314A or GFP gene, three types of plasmids (pLJM1-bCDwt-GFP, pLJM1-bCD-D314A-GFP and pLJM1-GFP) were transfected to 293T cells according to the instructions for Lipofectamine 2000™ (Invitrogen). The virus-containing supernatant was collected 48 h after transfection, concentrated by centrifugation (4000 r/mim, 4℃ for 5 min), and filtered with a 0.45-μm membrane filter. The virus titers were determined in 293T cells.

For LoVo cell infection, there were three groups: bCDwt-GFP, bCD-D314A-GFP, and GFP (null). LoVo cells were seeded at a density of 1×10^5 cells in a 60-mm plate and infected with different lentiviral vectors in the presence of 10 μg/mL polybrene (Millipore, Billerica, MA, USA). At 10-12 h post-infection, the growth medium was replaced. Forty-eight hours later, the GFP expression of transduced cells was observed under fluorescence microscopy. LoVo cells were infected twice in the same way. At 3 d after transfection, the FACSCalibur flow cytometer (BD, Franklin Lakes, NJ, USA) was used for fluorescence-activated cell sorting and then stable transfected cells, LoVo/null, LoVo/bCDwt and LoVo/bCD-D314A were cultured in a 5% CO_2-humidified incubator at 37℃.

Detection of bCD gene in transfected LoVo cells with RT-PCR

Total RNA was extracted from transfected LoVo cells harvested from the different groups with TRIzol reagent. First-strand cDNA was synthesized by reverse transcription according to the instructions for M2MLV (Promega, Madison, WI, USA). The sense sequence of bCD primers used in RT-PCR was 5'TTATGTCGAATAACGCTTTACAAAC-3', whereas the antisense sequence was 5'TACCTCCACGTTTGTAATCGATGGC-3'. PCR was performed for 35 cycles (94℃ for 1 min, 60℃ for 1.5 min, 72℃ for 1.5 min) in an automated DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA).

Xenograft tumor model study

To compare antitumor effects of bCDwt- and bCD-D314A-mediated molecular chemotherapy *in vivo*, combination of 5-FC, pools of LoVo, LoVo/null, LoVo/bCDwt or LoVo/bCD-D314A cells [5×10^6 cells in 100 μL PBS (pH 7.3)] were injected subcutaneously (s.c.) into the right flanks of 5-6-wk-old female Balb/c nude mice (n = 20, respectively). Two weeks post-LoVo cell inoculation, PBS or 5-FC (500 mg/kg) was administered by i.p. injection once daily for 14 d. Starting at day 1, the tumor volume was monitored daily using caliper measurement, calculated using the formula: $\pi/6 \times (\text{width} \times \text{length}^2)$. On the day after LoVo cell injection, mice were monitored daily for survival.

Statistical analysis

The treatment groups were compared with respect to tumor size. To test for significant differences in tumor volume among treatment groups, one-way ANOVA was conducted. When ANOVA indicated that a significant difference existed ($P < 0.05$), multiple comparison procedures were used to determine where the differences lay. Kaplan-Meier survival curves were analyzed by the log-rank test, and specific pairwise multiple comparisons were made using the Holm-Sidak method. All comparisons were made using the 0.05 level of significance.

RESULTS

Identification of recombinant lentiviral plasmids

Sequencing results of recombinant lentiviral plasmids showed that pLJM1-bCDwt-GFP contained the wild-type bCD gene, and pLJM1-bCD-D314A-GFP contained the mutant D314A (Figure 1), which indicated that the two recombinant lentiviral plasmids were constructed successfully.

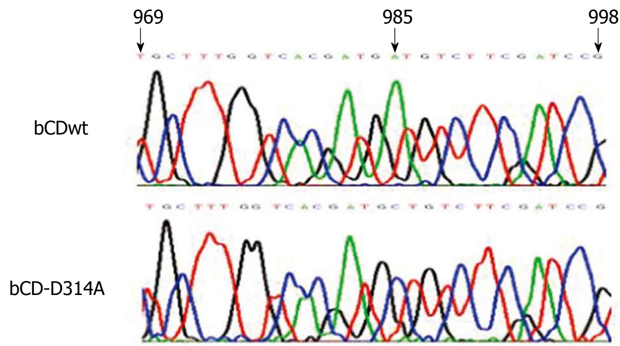


Figure 1 Sequencing results of recombinant plasmid pLJM1-bCDwt and pLJM1-bCD-D314A. The codon 985 of bCDwt, A, was mutated to C in bCD-D314A, which was the mutant D314A of bCD.

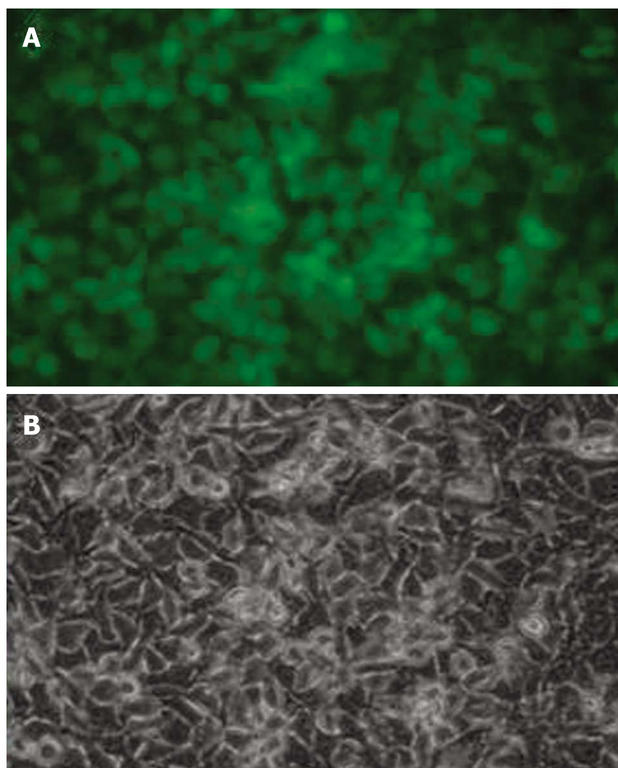


Figure 2 LoVo cell with fluorescence after transfection with lentiviral vector containing bCDwt-GFP gene (× 100). A: Green fluorescence field; B: Visible light field.

Efficacy of gene delivery by lentiviral vectors

Lentiviral vectors have improved efficiency to deliver genes. In this study, GFP was used as a reporter gene. Figure 2 shows that the efficacy of gene delivery by lentiviral vectors was satisfactory.

Identification of bCD gene in LoVo cells transfected with lentiviral vectors

LoVo cells transfected with different lentiviral vectors, LoVo/null, LoVo/bCDwt and LoVo/bCD-D314A, were subjected to RT-PCR to identify bCD gene expression. As shown in Figure 3, LoVo/bCDwt and LoVo/bCD-D314A cells had bCD gene expression, while bCD gene was not detected in LoVo/null cells.

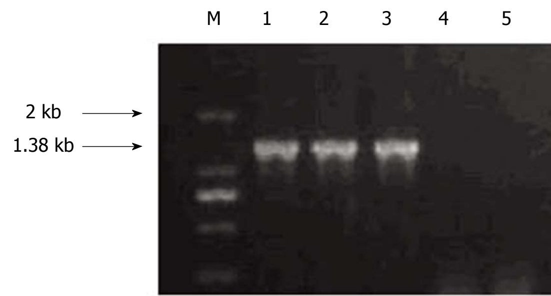


Figure 3 Identification of bCD gene in LoVo cells transfected with different lentiviral vectors. M: Marker; 1: Positive control (pcDNA3.1/bCDwt); 2: LoVo/bCDwt; 3: LoVo bCD-D314A; 4: LoVo/null; 5: Negative control (LoVo).

Antitumor effects of bCDwt and bCD-D314A in combination with 5-FC in human colon carcinoma xenograft model

To evaluate the potential of bCD-D314A gene therapy with 5-FC *in vivo*, LoVo cells with different stably gene delivery were injected s.c. into the right flank of athymic nude mice. Two weeks after cell inoculation, before treatment, the tumors in each group were palpable and the mean volumes in each group did not differ significantly among the treatment groups ($P > 0.05$), and within treatment variances (PBS *vs* 5-FC) were not significantly different ($P > 0.05$). The baseline mean tumor volume at 14 d after tumor cell injection was 412.63 ± 36.79 mm^3.

PBS or 5-FC (500 mg/kg) was administered i.p. once daily for 2 wk. Starting at day 1, mice were monitored for tumor volume and survival. Inhibition of tumor growth was initially noted in mice treated with LoVo/bCDwt or LoVo/bCD-D314A in combination with 5-FC compared with the other groups on day 20 ($P < 0.05$) (Figure 4). There were no significant differences in tumor growth between the other groups ($P > 0.05$), which indicated that gene delivery or 5-FC administration alone had no influence on tumor growth. From day 20 onwards, tumors in the mice treated with LoVo/bCD-D314A and 5-FC shrunk daily, whereas the tumors in mice treated with LoVo/bCDwt and 5-FC increased gradually. The difference in tumor volume between these two groups became increasingly marked ($P < 0.05$). At the same time, the tumors in the other groups kept growing (Figure 4).

As to the influence of bCD-D314A and bCDwt on survival, we showed that bCD-D314A/5-FC or bCDwt/5-FC treatment significantly prolonged survival of mice in comparison with the other groups. As shown in Figure 5, the median survival time of other groups was about 35 d and there was no difference among them ($P > 0.05$), whereas it was prolonged to 62 or 94 d in the bCDwt/5-FC or bCD-D314A/5-FC group, respectively ($P < 0.05$). Furthermore, bCD-D314A plus 5-FC significantly prolonged survival of mice in comparison with bCDwt plus 5-FC ($P < 0.05$).

DISCUSSION

Worldwide, more than one million individuals will develop colorectal cancer annually, and the disease-specific mortal-

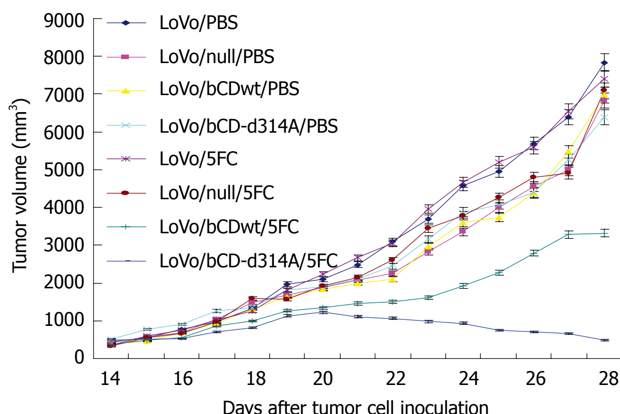


Figure 4 Growth of LoVo, LoVo/null, LoVo/bCDwt, and LoVo/bCD-D314A xenografts treated with PBS or 5-fluorocytosine. Treatment was started on 14 d after tumor cell inoculation. PBS or 5-fluorocytosine (5-FC) (500 mg/kg) was injected intraperitoneal once a day for 14 d. Data points represent the mean tumor volume of each group of animals. bCD: Bacterial cytosine deaminase; bCDwt: Wild-type bCD; bCD-D314A: bCD mutant D314A; LoVo/null: LoVo cell transfected with pLJM1-GFP; LoVo/bCDwt: LoVo cell transfected with pLJM1-bCDwt-GFP; LoVo/bCD-D314A: LoVo cell transfected with pLJM1-bCD-D314A-GFP.

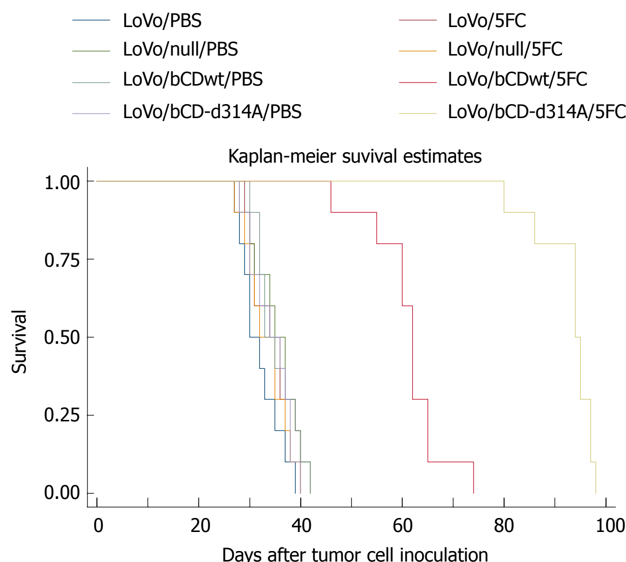


Figure 5 Efficacy of wild-type bCD gene or bCD mutant D314A gene suicide gene therapy in human colon carcinoma xenografts. LoVo human colon carcinoma cells with different gene transfection (5 × 10^6 cells/mouse) were injected into the right flank of athymic nude mice (10 mice/group). Two weeks after tumor cell injection, PBS or 5-fluorocytosine (5-FC) (500 mg/kg) was injected intraperitoneal once a day for 14 d. On the day after LoVo cell injection, mice were monitored daily for survival. bCD: Bacterial cytosine deaminase; bCDwt: wild-type bCD; bCD-D314A: bCD mutant D314A; LoVo/null: LoVo cell transfected with pLJM1-GFP; LoVo/bCDwt: LoVo cell transfected with pLJM1-bCDwt-GFP; LoVo/bCD-D314A: LoVo cell transfected with pLJM1-bCD-D314A-GFP.

ity rate is nearly 33% in the developed world[1]. Substantial progress has been made in understanding the molecular pathogenesis, diagnosis (hereditary and sporadic), and treatment of colorectal cancer during recent decades. Despite the use of 5-FU-based combination chemotherapy and active targeted drugs for treatment of metastatic colorectal cancer in the past decade, and improvement in overall survival for non-resectable disease, cure rates remain low[25]. There is a need for the development of new alternative therapeutic strategies. Gene therapy is a novel approach that might lead to improved treatments for colorectal cancer. Among these approaches, GDEPT using the bCD/5-FC system has been developed. The gene that encodes the CD that converts the prodrug 5-FC to 5-FU is delivered to the target tumor cells, which results in their death.

The most important characteristic of suicide gene therapy is its bystander effect. Although the viral or non-viral gene delivery systems currently available have poor efficacy for *in vivo* gene transfer, complete eradication of tumors has been seen in some experimental animal models, which is thought to depend on the bystander killing effect. In the bCD/5-FC system, the bystander effect is caused by the passive diffusion of 5-FU into the extracellular milieu and its diffusion into the adjacent cells, which requires no gap junctions[11]. The immune-related response also contributes to the bystander effect[26], which has been confirmed by our experimental results from the immunocompetent and immunodeficient mice[27]. Although this approach has been in development for several decades, new combinations with cancer therapies, such as selective conventional chemotherapy[28] and radiotherapy[29], are being tested.

Unlike conventional chemotherapy, suicide gene therapy renders specific killing of the tumor cells that express the suicide gene, but it may lead to systemic toxicity if these genes are delivered to normal cells. Thus, target specificity is of great importance to suicide gene therapy. The rationale behind suicide gene therapy is that, after targeted transfer of these genes into tumor cells, only tumor and neighboring cells will be rendered sensitive to their cytotoxic action. Specifically, targeted expression of the prodrug-activating enzyme avoids systemic toxicity, and results in high drug concentrations in the tumor mass and an improved therapeutic index compared with non-targeted gene delivery. To kill carcinoembryonic antigen (CEA)-positive colorectal carcinoma cells specifically using the bCD/5-FC system, we have constructed a new replication-deficient recombinant adenoviral vector that contains the bCD gene controlled by the CEA promoter, AdCEACD, and have evaluated its *in vitro* cytotoxic effects. We have shown that this vector can transfer bCD to CEA-positive tumor cells specifically by comparing the vector with cytomegalovirus (CMV) promoter, AdCMVCD[7]. However, the cytotoxic effects of bCD/5-FC decreased to some extent[22,23]. Although this loss of activity may be due to differences in transcriptional activation between the CEA and CMV promoters, the low affinity displayed by wild-type bCD towards 5-FC in comparison with cytosine is thought to be the principal factor that leads to the relatively poor turnover of 5-FC of wild-type bCD and limits the overall therapeutic response.

It has been shown previously that the bCD mutant, D314A, decreased efficiency for endogenous cytosine, which can compete with the prodrug for the active enzyme site, in combination with increased efficiency for 5-FC that resulted in a 19-fold relative substrate preference for 5-FC in comparison with bCDwt[16,19]. The bCD mutant D314A has been demonstrated to be an excellent candidate for

subsequent preclinical comparisons with wild-type bCD and yCD.

Recently, we have constructed the bCD mutant D314A using site-directed mutagenesis. The *in vitro* results have indicated that its killing and bystander effects on human colon cancer LoVo cells are enhanced significantly as compared with wild-type bCD[24]. Thus, the rationale for using the mutant bCD gene for colon carcinoma *in vivo* is that the bCD mutant D314A can more effectively convert 5-FC to 5-FU, and increase the antitumor activity and prolong survival.

In the present study, we investigated mutant bCD gene transfer with lentiviral vector for treatment of human colon cancer in xenograft models. Lentivirus-based vectors (lentivectors) have been developed with improved efficiency, specificity, and safety, and are being increasingly used in basic and applied research. Clinical trials of human gene therapy are currently underway using lentivectors in a wide range of human diseases[30]. In the present study, lentiviral vector was used to transfer suicide genes. These preliminary results confirmed the efficacy of lentiviral vector for suicide gene delivery.

After the LoVo cells stably transfected with bCDwt gene or mutant bCD-D314A gene were established, they were inoculated into athymic nude mice to produce xenograft tumor models. Afterwards, 5-FC was administered. As expected, a more potent cytotoxicity effect for colon cancer was obtained using bCD-D314A/5-FC treatment in comparison with bCDwt/5-FC. During 5-FC administration, the tumors treated with bCDwt/5-FC or bCD-D314A/5-FC grew slower than those in other treatment groups, which indicated that 5-FC or suicide gene transfer alone had no effect on colon cancer. The comparative study of bCD-D314A/5-FC and bCDwt/5-FC showed an increased antitumor effect, and decreased tumor growth was observed following bCD-D314A/5-FC gene therapy. Furthermore, survival analysis showed that bCD-D314A/5-FC therapy prolonged life significantly, which confirmed the enhanced antitumor activity of bCD mutant D314A.

Although the intratumoral or blood 5-FU concentration was not estimated after 5-FC administration in this study, the enhanced antitumor effect of bCD mutant D314A in combination with 5-FC was thought to be due to its ability to convert 5-FC to 5-FU more effectively, which is consistent with recently published data in other tumor models[20,21]. The blood and tumor levels of 5-FC and 5-FU are a subject for future studies that will enable a rational dosing strategy.

In summary, our studies provide preliminary evidence that treatment using bCD mutant D314A for suicide gene/5-FC prodrug therapy is a promising approach for treatment of human colon carcinoma. Further studies on delivery systems, doses and protocols would be worthwhile to optimize this approach.

ACKNOWLEDGMENTS

The authors thank Professor Jian-Ming Li for the kind gift of pLJM1.

COMMENTS

Background

Suicide gene therapy is an appealing alternative to conventional therapies for colorectal cancer. Bacterial cytosine deaminase (bCD) can convert the non-toxic prodrug 5-fluorocytosine (5-FC) to its toxic form, 5-fluorouracil (5-FU). However, 5-FC is a poor substrate for bCD compared with its native substrate, cytosine, and its antitumor effect is limited.

Research frontiers

The bCD mutant, D314A, has been shown to prefer 5-FC over cytosine by nearly 20-fold, and has been demonstrated to enhance therapeutic efficacy against human glioma and human pancreatic tumors. However, there have been only a few studies of bCD mutants in colorectal cancer.

Innovations and breakthroughs

Recently, the authors have constructed the bCD mutant, D314A, using site-directed mutagenesis, and have demonstrated that D314A has significantly increased cytotoxicity on human colon cancer cell line LoVo compared with wild-type bCD (bCDwt). The present study indicated that mutant D314A was able to significantly enhance antitumor efficacy in human colon cancer xenograft models compared with bCDwt.

Applications

Collectively, these studies provide preliminary evidence that treatment using bCD mutant D314A for suicide gene/5-FC prodrug therapy provides a promising approach for human colon carcinoma.

Terminology

Suicide gene therapy is a form of gene-directed enzyme-prodrug therapy. When the suicide gene is delivered to cancer cells and a non-toxic prodrug is administered, the enzyme converts the non-toxic prodrug into its active and lethal form, which results in cancer cell death.

Peer review

The authors investigated whether bCD mutant D314A suicide gene and 5-FC prodrug therapy increased therapeutic efficacy in a nude mouse model of human colon cancer, using lentiviral vectors. It revealed for the first time that D314A significantly enhanced antitumor activity in human colon cancer xenograft models. The results are useful and may provide a new strategy to treat colorectal cancer.

REFERENCES

1 **Jemal A**, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin* 2010; **60**: 277-300

2 **Weiss J**, Moghanaki D, Plastaras JP, Haller DG. Improved patient and regimen selection in locally advanced rectal cancer: who, how, and what next? *Clin Colorectal Cancer* 2009; **8**: 194-199

3 **Sharma A**, Tandon M, Bangari DS, Mittal SK. Adenoviral vector-based strategies for cancer therapy. *Curr Drug ther* 2009; **4**: 117-138

4 **Wu C**, Lin J, Hong M, Choudhury Y, Balani P, Leung D, Dang LH, Zhao Y, Zeng J, Wang S. Combinatorial control of suicide gene expression by tissue-specific promoter and microRNA regulation for cancer therapy. *Mol Ther* 2009; **17**: 2058-2066

5 **Figueiredo ML**, Kao C, Wu L. Advances in preclinical investigation of prostate cancer gene therapy. *Mol Ther* 2007; **15**: 1053-1064

6 **Chaszczewska-Markowska M**, Stebelska K, Sikorski A, Madej J, Opolski A, Ugorski M. Liposomal formulation of 5-fluorocytosine in suicide gene therapy with cytosine deaminase-for colorectal cancer. *Cancer Lett* 2008; **262**: 164-172

7 **Shen LZ**, Wu WX, Xu DH, Zheng ZC, Liu XY, Ding Q, Hua YB, Yao K. Specific CEA-producing colorectal carcinoma cell killing with recombinant adenoviral vector containing cytosine deaminase gene. *World J Gastroenterol* 2002; **8**: 270-275

8 **Yazawa K**, Fisher WE, Brunicardi FC. Current progress in suicide gene therapy for cancer. *World J Surg* 2002; **26**: 783-789

9 **Huang Q**, Liu XZ, Kang CS, Wang GX, Zhong Y, Pu PY. The anti-glioma effect of suicide gene therapy using BMSC expressing HSV/TK combined with overexpression of Cx43 in glioma cells. *Cancer Gene Ther* 2010; **17**: 192-202

10 **Garcia-Rodríguez L**, Abate-Daga D, Rojas A, González JR,

Fillat C. E-cadherin contributes to the bystander effect of TK/GCV suicide therapy and enhances its antitumoral activity in pancreatic cancer models. *Gene Ther* 2011; **18**: 73-81

11 **Spasojević I**, Maksimović V, Zakrzewska J, Bacić G. Effects of 5-fluorouracil on erythrocytes in relation to its cardiotoxicity: membrane structure and functioning. *J Chem Inf Model* 2005; **45**: 1680-1685

12 **Brown NL**, Lemoine NR. Clinical trials with GDEPT: cytosine deaminase and 5-fluorocytosine. *Methods Mol Med* 2004; **90**: 451-457

13 **Freytag SO**, Stricker H, Pegg J, Paielli D, Pradhan DG, Peabody J, DePeralta-Venturina M, Xia X, Brown S, Lu M, Kim JH. Phase I study of replication-competent adenovirus-mediated double-suicide gene therapy in combination with conventional-dose three-dimensional conformal radiation therapy for the treatment of newly diagnosed, intermediate- to high-risk prostate cancer. *Cancer Res* 2003; **63**: 7497-7506

14 **Breton E,** Goetz C, Kintz J, Accart N, Aubertin G, Grellier B, Erbs P, Rooke R, Constantinesco A, Choquet P. In vivo preclinical low-field MRI monitoring of tumor growth following a suicide-gene therapy in an orthotopic mice model of human glioblastoma. *C R Biol* 2010; **333**: 220-225

15 **Shi DZ**, Hu WX, Li LX, Chen G, Wei D, Gu PY. Pharmacokinetics and the bystander effect in CD::UPRT/5-FC bi-gene therapy of glioma. *Chin Med J* (Engl) 2009; **122**: 1267-1272

16 **Mahan SD**, Ireton GC, Stoddard BL, Black ME. Alanine-scanning mutagenesis reveals a cytosine deaminase mutant with altered substrate preference. *Biochemistry* 2004; **43**: 8957-8964

17 **Diasio RB**, Lakings DE, Bennett JE. Evidence for conversion of 5-fluorocytosine to 5-fluorouracil in humans: possible factor in 5-fluorocytosine clinical toxicity. *Antimicrob Agents Chemother* 1978; **14**: 903-908

18 **Stolworthy TS**, Korkegian AM, Willmon CL, Ardiani A, Cundiff J, Stoddard BL, Black ME. Yeast cytosine deaminase mutants with increased thermostability impart sensitivity to 5-fluorocytosine. *J Mol Biol* 2008; **377**: 854-869

19 **Mahan SD**, Ireton GC, Knoeber C, Stoddard BL, Black ME. Random mutagenesis and selection of Escherichia coli cytosine deaminase for cancer gene therapy. *Protein Eng Des Sel* 2004; **17**: 625-633

20 **Kaliberov SA**, Market JM, Gillespie GY, Krendelchtchikova V, Della Manna D, Sellers JC, Kaliberova LN, Black ME, Buchsbaum DJ. Mutation of Escherichia coli cytosine deaminase significantly enhances molecular chemotherapy of human glioma. *Gene Ther* 2007; **14**: 1111-1119

21 **Kaliberova LN**, Della Manna DL, Krendelchtchikova V, Black ME, Buchsbaum DJ, Kaliberov SA. Molecular chemotherapy of pancreatic cancer using novel mutant bacterial cytosine deaminase gene. *Mol Cancer Ther* 2008; **7**: 2845-2854

22 **Shen LZ**, Hua YB, Wu WX, Xu DH, Ding Q, Liu XY, Wang GL. IL-2 gene therapy enhances cytotoxic effect of E. Coli. Cytosine deaminase gene for colon cancer. *Zhonghua Weichang Waike Zazhi* 2004; **7**:411-413

23 **Shen LZ**, Hua YB, Wu WX, Xu DH, Ding Q, Chen GY, Zheng ZC, Liu XY. Enhancement of tumor killing using a combination of E. Coli. Cytosine deaminase gene and INF- gene therapy. *Acta Univ Med Nanjing* 2004; **24**: 618-620

24 **Sun MC**, Huang YM, Zhu ZC, Wang JP, Shen LZ, Wu WX. Inhubitory effect of mutant cytosine deaminase D314A against huaman colon cancer cells. *Zhongguo Zhongliu Shengwu Zhiliao Zazhi* 2009; **16**: 595-599

25 **Cunningham D**, Atkin W, Lenz HJ, Lynch HT, Minsky B, Nordlinger B, Starling N. Colorectal cancer. *Lancet* 2010; **375**: 1030-1047

26 **Agard C**, Ligeza C, Dupas B, Izembart A, El Kouri C, Moullier P, Ferry N. Immune-dependent distant bystander effect after adenovirus-mediated suicide gene transfer in a rat model of liver colorectal metastasis. *Cancer Gene Ther* 2001; **8**: 128-136

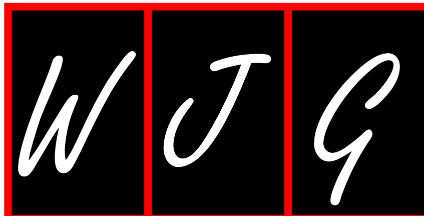
27 **Shen LZ**, Wu WX, Hua YB, Ding Q, Chen T. The relationship between the bystander effect of E. coli cytosine deaminase gene depends on the immune status of host. *Linchuang Zhongliuxue Zazhi* 2004; **9**: 237-240

28 **Shen LZ**, Ding Q, Wu WX, Xu DH, Liu XY, Zheng ZC, Wu ZY. In vitro effect of cytosine deaminase gene therapy and chemical reagents on colon cancer cell line. *Zhonghua Weichang Waike Zazhi* 2002; **17**: 404-406

29 **Xing L**, Sun X, Deng X, Kotedia K, Urano M, Koutcher JA, Ling CC, Li GC. Expression of the bifunctional suicide gene CDUPRT increases radiosensitization and bystander effect of 5-FC in prostate cancer cells. *Radiother Oncol* 2009; **92**: 345-352

30 **Escors D**, Breckpot K. Lentiviral vectors in gene therapy: their current status and future potential. *Arch Immunol Ther Exp* (Warsz) 2010; **58**: 107-119

S- Editor Sun H **L- Editor** Kerr C **E- Editor** Ma WH


World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2965

World J Gastroenterol 2011 June 28; 17(24): 2965-2971
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2011 Baishideng. All rights reserved.

BRIEF ARTICLE

CD133$^+$ gallbladder carcinoma cells exhibit self-renewal ability and tumorigenicity

Cheng-Jian Shi, Jun Gao, Min Wang, Xin Wang, Rui Tian, Feng Zhu, Ming Shen, Ren-Yi Qin

Cheng-Jian Shi, Min Wang, Xin Wang, Rui Tian, Feng Zhu, Ming Shen, Ren-Yi Qin, Department of Biliary-pancreatic Surgery, Affiliated Tongji Hospital, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China
Jun Gao, Department of General Surgery, The Affiliated Hospital of Qingdao University Medical College, Qingdao 266003, Shandong Province, China
Author contributions: Shi CJ and Gao J contributed equally to this work; Shi CJ, Gao J and Wang X performed the majority of the experiments, analyzed the data and wrote the manuscript; Tian R and Qin RY designed the research and drafted the manuscript; Wang X, Wang M and Shen M helped edit the manuscript; Zhu F contributed to the cell culture experiments.
Supported by Grant from the National Natural Science Foundation of China, No. 30772172
Correspondence to: Ren-Yi Qin, MD, Professor, Department of Biliary-pancreatic Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, No. 1095, Jiefang Ave., Wuhan 430030, Hubei Province, China. ryqin@tjh.tjmu.edu.cn
Telephone: +86-27-83663814 Fax: +86-27-83663874
Received: October 25, 2010 Revised: November 28, 2010
Accepted: December 5, 2010
Published online: June 28, 2011

Abstract

AIM: To identify cancer stem cells (CSCs) in human gallbladder carcinomas (GBCs).

METHODS: Primary GBC cells were cultured under serum-free conditions to produce floating spheres. The stem-cell properties of the sphere-forming cells, including self-renewal, differentiation potential, chemoresistance and tumorigenicity, were determined *in vitro* or *in vivo*. Cell surface expression of CD133 was investigated in primary tumors and in spheroid cells using flow cytometry. The sphere-colony-formation ability and tumorigenicity of CD133$^+$ cells were assayed.

RESULTS: *In vitro* culture experiments revealed that floating spheroids were generated from primary GBC cells, and these sphere-forming cells could generate new progeny spheroids in serum-free media. Spheroid cells were differentiated under serum-containing conditions with downregulation of the stem cell markers Oct-4, Nanog, and nestin ($P < 0.05$). The differentiated cells showed lower spheroid-colony-formation ability than the original spheroid cells ($P < 0.05$). Spheroid cells were more resistant to chemotherapeutic reagents than the congenetic adherent cells ($P < 0.05$). Flow cytometry showed enriched CD133$^+$ population in sphere-forming cells ($P < 0.05$). CD133$^+$ cells possessed high colony-formation ability than the CD133$^-$ population ($P < 0.01$). CD133$^+$ cells injected into nude mice revealed higher tumorigenicity than their antigen-negative counterparts ($P < 0.05$).

CONCLUSION: CD133 may be a cell surface marker for CSCs in GBC.

© 2011 Baishideng. All rights reserved.

Key words: Gallbladder carcinoma; Cancer stem cell; Non-adherent spheres; CD133 protein; Self-renewal; Tumorigenicity

Peer reviewer: Giedrius Barauskas, Professor, Department of Surgery, Kaunas University of Medicine, Eiveniu str. 2, Kaunas, LT-50009, Lithuania

Shi CJ, Gao J, Wang M, Wang X, Tian R, Zhu F, Shen M, Qin RY. CD133$^+$ gallbladder carcinoma cells exhibit self-renewal ability and tumorigenicity. *World J Gastroenterol* 2011; 17(24): 2965-2971 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2965.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2965

INTRODUCTION

Gallbladder carcinoma (GBC) is the most common

malignant neoplasm of the biliary tract and the seventh most common gastrointestinal cancer[1]. Its clinical presentation is nonspecific and may include abdominal pain, weight loss, fever, and jaundice. Current evidence suggests that radical surgery is the only curative treatment for GBC. However, despite development in surgery, the 5-year survival rate in patients with advanced stage GBC is still only around 10%[2,3]. Pooling of carcinogens under conditions causing biliary stasis, or malignant degeneration of metaplastic changes after chronic inflammation have been suggested as possible factors, but the precise pathogenetic mechanisms of GBC remain unclear[1]. The biology of GBC therefore needs further investigation.

Emerging evidence has shown that the abilities for tumor growth and propagation reside in a small population of tumor cells, termed cancer stem cells (CSCs) or tumor-initiating cells. These cells possess properties of self-renewal, differentiation potential, resistance to chemotherapy, and high tumorigenicity[4-8]. Based on this hypothesis, CSCs were initially isolated from human acute myeloid leukemia[9]. Regarding solid tumors, the existence of CSCs in breast cancer was reported in 2003, when as few as 200 $CD44^+CD24^{-/low}$ ESA^+ breast cancer cells were shown to be adequate to produce new tumors in nonobese diabetic/severe combined immunodeficient mice, whereas a significantly higher number of other cell populations failed to form tumor xenografts[10]. Tumor-initiating cells with distinct cell surface markers have recently been identified in various solid tumors, such as brain[11], prostate[12], pancreatic[13], and ovarian cancer[14], and in Ewing's sarcoma[15]. It is generally considered that the identification of the CSCs could have a significant impact on the understanding of tumor biology and therapy.

Several different methods have previously been used to identify CSCs[16,17], including the culture of cancer cells under non-adherent conditions in serum-free media containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). The growth of spherical colonies is considered to reflect the self-renewal ability and phenotype of CSCs. In the present study, we cultured primary GBC cells to generate spherical colonies and estimated their differentiation potential in the serum medium. We compared the chemoresistance of spheroid cells and differentiated cells *in vitro*. We also examined the expression of the CSC surface marker CD133, and investigated its use as a candidate marker to further identify the CSC phenotype in GBC, including comparing the *in vitro* spheroid-colony-formation and *in vivo* tumorigenicity of $CD133^+$ and $CD133^-$ cells. The results of this study may clarify the phenotype of CSCs in GBC, thus contributing to the development of more effective therapeutic approaches.

MATERIALS AND METHODS

Preparation of single cancer cells

Two samples of human GBC were obtained after surgical excision in accordance with Institutional Review Board-approved guidelines. Tumor tissue specimens were dissociated using scissors and scalpels, mixed with collagenase Ⅳ (Invitrogen, USA) in medium199 (collagenase 200 U/mL, Invitrogen), and incubated at 37℃ for 2.5-3 h. At the end of the incubation, cells were filtered through a 40-μm nylon mesh and washed twice with phosphate-buffered saline (PBS)/10% fetal bovine serum (FBS, Gibco, USA).

Tumor cell cultures

The single tumor cells were suspended in serum-free DMEM/F12 (1:1 volume, Gibco) consisting of 20 ng/mL human recombinant EGF (PeproTech, USA), 20 ng/mL bFGF (PeproTech), 5 μg/mL insulin (Sigma, USA), and cultured in 24-well culture plates at a density of 1×10^4/well. Fresh serum-free DMEM/F12 (described above) was added into the wells at 0.05 mL/well every day. Spheroids were collected and dissociated 2 wk after primary culture. The resulting single cells were placed into stem cell culture medium to generate progeny spheres. Images of the spheroid colonies were recorded using an inverted microscope (Nikon, Type 108) equipped with a Nikon 2000-S Inverted Photomicroscope and Nikon NIS-Elements F2.30 software.

Differentiation assay

To assess their differentiation potential, spheres were collected and placed into DMEM/F12 supplemented with 10% FBS without growth factors, as described previously, and cell morphology was observed. After 14 d of culture in differentiating medium, tumor cells were collected and suspended in serum-free DMEM/F12 (described above), and cultured in 96-well culture plates at a density of 10 cells per well. Fresh serum-free DMEM/F12 was added into the wells at 0.025 mL per well every day. After 2 wk, each well was examined under light microscope and the total number of spheroid colonies in the 96-well plates was counted.

Real-time quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from spheroid cells or adherent cells using RNeasy Mini kit (Qiagen), and was reverse transcribed into cDNA using M-MLV reverse transcriptase enzyme (Sigma). Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using an ABI PRISM 7900HT sequence detection system (Applied Biosystems), according to the manufacturer's instructions. The relative mRNA expression levels of the tested genes were normalized to the level of endogenous control gene, glyceraldehyde-3-phosphate dehydrogenase.

Chemoresistance experiments

Cells were seeded in 96-well plates at 3000 cells per well. Each well was supplied with DMEM medium containing 10% FBS, together with either gemcitabine (1 μg/mL, Sigma) or 5-fluorouracil (0.1 μg/mL, Sigma), or no drug as control. The culture medium was changed 3 d after

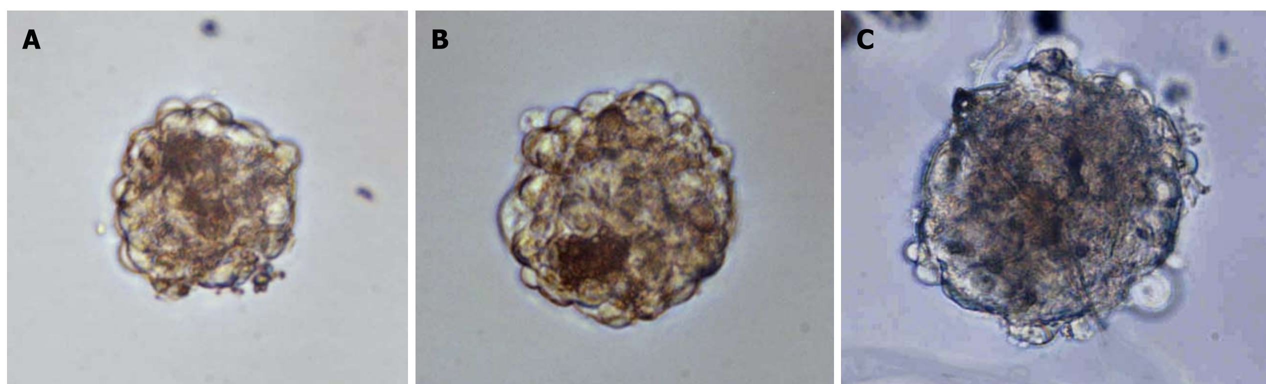


Figure 1 Culture of floating spheres. Single primary gallbladder carcinoma cells were cultured in serum-free medium containing human epidermal growth factor (20 ng/mL) and basic fibroblast growth factor (10 ng/mL). A: After culture for 1 wk, non-adherent spheres were observable (original magnification × 200); B: The spheres were dissociated and were plated into the same stem-cell-selective medium; similar progeny spheres emerged after 2 wk (original magnification × 200); C: The second progeny spheres were derived from the first progeny sphere-forming cells (original magnification × 200).

initial treatment and the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, 20 μL of MTT (5 mg/mL in PBS, Sigma) was added to the medium for 4 h. Medium and MTT were removed, dimethylsulfoxide (Sigma) was added, and the absorbance was measured at 490 nm using a plate reader Multiskan EX (Thermo Fisher Scientific Inc., Waltham, MA).

Detection of CD133 expression using flow cytometry

Cells derived from primary tumors or spheres were separately resuspended in PBS with 2% FBS at a concentration of 10^6/100 μL. Anti-CD133/1-phycoerythrin (eBioscience, USA) was added to the samples, and incubated on ice for 30 min. After incubation, the samples were washed twice with 2% FBS/PBS and resuspended in 2% FBS/PBS. Flow cytometric analysis was performed using a FACSAria (BD Immunocytometry Systems, Franklin Lakes, NJ, USA).

Spheroid-colony-formation assay of $CD133^+$ GBC cells

$CD133^+$ and $CD133^-$ populations were sorted from sphere-forming cells using fluorescence-activated cell sorting (FACS). For FACS, cells were collected and stained, and sorted using a FACSAria. The sorted tumor cells were suspended in serum-free DMEM/F12, and cultured in 96-well culture plates at a density of 10/well. After 2 wk, the total number of spheroid colonies in the 96-well plate was counted, as described above.

Tumorigenicity of $CD133^+$ GBC cells in vivo

Female nude mice (BALB/C), 4-6 wk old, were purchased from Hunan Slack King of Laboratory Animal Co., Ltd. (Changsha, China). $CD133^+$ and $CD133^-$ populations were sorted from two primary tumors (tumor 3 and tumor 4), and from sphere-forming cells using FACS. Cells were routinely sorted twice, and reanalyzed for a purity, which was typically > 90%. Sorted cells were resuspended in PBS/Matrigel mixture (1:1 volume). The mice were anesthetized using ethyl ether and 10 000 tumor cells were injected subcutaneously into the abdominal region, using a gauge needle. The mice were maintained under standard conditions according to the institutional guidelines for animal care. Tumor appearance was inspected weekly by visual observation and palpation. Animal experiments were terminated 3 mo after cell injection.

Hematoxylin and eosin staining and immunohistochemistry

Primary tumor tissues and mouse xenografts were fixed in 10% formalin and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin (HE) to assess tumor type. The sections were incubated with anti-human CA19-9 antibody (Abcam, UK) and secondary antibodies using an ImmunoPure ABC Staining kit (Santa Cruz, USA), according to standard immunohistochemical procedures. Negative controls containing no primary antibody were prepared. All microscopic images were captured as above.

Statistical analysis

Data were expressed as mean ± SD and Student's *t* test was used to compare the differences between groups. Values of $P < 0.05$ were considered significant.

RESULTS

Spheroid formation

Previous studies indicated that CSCs could produce floating three-dimensional tumor spheroids under stem-cell-selective conditions[18-21]. Based on these studies, we cultured primary human GBC cells in serum-free DMEM/F12 in an attempt to expand human GBC CSCs. Non-adherent spheres derived from human GBCs were observable after *in vitro* culture for 1 wk (Figure 1A), and these continued to expand for 2-3 wk in serum-free media. The spheres were dissociated and the resulting single cells were plated in the same stem-cell-selective medium; similar progeny spheres emerged after 2 wk (Figure 1B and C). This demonstrated that tumor sphere cells had self-renewing characteristics.

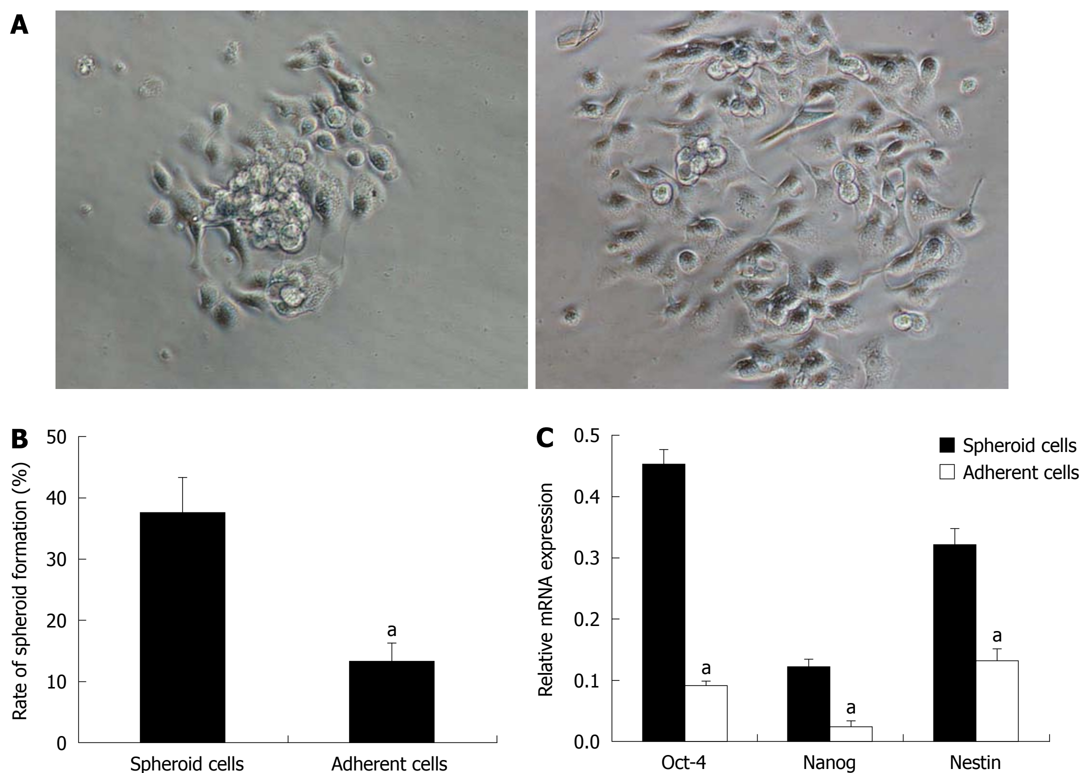


Figure 2 Differentiation of spheroid cells. Non-adherent spheres were collected and placed into DMEM/F12 supplemented with 10% fetal bovine serum. A: After 6 h, cells migrated from the spheres and became adherent and the sphere volume was significantly reduced (original magnification × 200); B: The spheroid-colony-forming ability of spheroid cells decreased under differentiating conditions. [a]$P < 0.05$ *vs* spheroid cells under stem-cell-selective conditions; C: The expression levels of Oct-4, Nanog and nestin were examined using real-time quantitative reverse transcription-polymerase chain reaction. The stem cell markers were downregulated in the adherent cells. [a]$P < 0.05$ *vs* spheroid cells under stem-cell-selective conditions.

Sphere-forming cells displayed differentiation potential

Spheres were cultivated under differentiating conditions to determine the differentiation potential of the tumor sphere cells. After 6 h of culture, the floating tumor spheres attached to the bottom of the culture plates and cells migrated from the spheres and became adherent (Figure 2A). After 14 d of culture in differentiating conditions, the sphere-formation ability of the adherent cells was assayed. The spheroid-colony-forming ability decreased, compared with that of the original sphere-forming cells ($P < 0.05$, Figure 2B). The expression of stem cell markers, including Oct-4, Nanog and nestin, were examined using real-time RT-PCR. These markers indicate an undifferentiated stem cell phenotype[22,23]. Spheroid cells showed higher expression of these markers than adherent cells ($P < 0.05$, Figure 2C), strongly supporting the idea that spheroid cells were differentiated in serum-containing medium.

Sphere-forming cells displayed high chemoresistance in vitro

Previous studies suggested that CSCs in several solid tumors possessed higher chemoresistance than non-CSCs[24-26]. To examine if our spheroid cells also possessed a CSC chemoresistant phenotype, the chemosensitivities of these cells were assessed under stem-cell-selective *vs* differentiating conditions. Spheroid cells under stem-cell-selective conditions displayed a greater resistance to gemcitabine and 5-fluorouracil than those under differentiating conditions ($P < 0.05$, Figure 3).

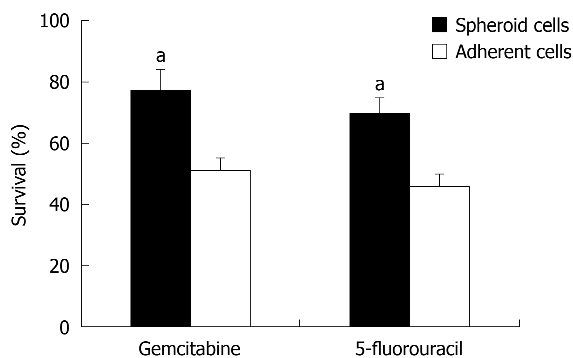


Figure 3 Chemoresistance assays of spheroid cells. Sphere-forming cells and differentiated cells were seeded in 96-well plates at 3000 cells/well. Chemotherapeutic reagents gemcitabine (1 μg/mL) and 5-fluorouracil (0.1 μg/mL) were added and cell survival was estimated by 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide assay. [a]$P < 0.05$ *vs* differentiated cells.

CD133+ cells were enriched in tumor spheres

The expression pattern of a possible candidate cell surface marker for CSCs was examined in primary human GBC and in sphere-forming cells, using flow cytometry. CD133 was selected as a potential marker, based on the results of previous studies of CSCs in solid tumors. Flow cytometric analysis revealed that CD133+ cells were pres-

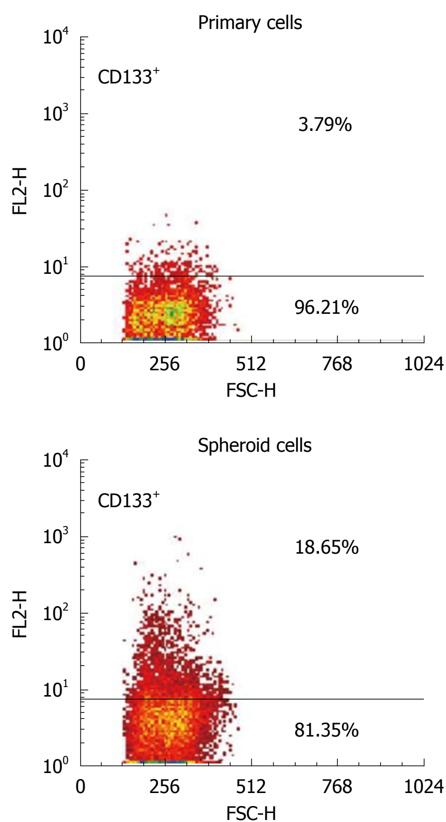


Figure 4 CD133 expression in gallbladder carcinoma spheroid cells. The single cells dissociated from primary tumors or spheres were incubated with anti-CD133/1-phycoerythrin and flow cytometric analysis was performed. The percentage of CD133+ cells was higher in the spheroid cells. P < 0.05 *vs* primary gallbladder carcinoma cells.

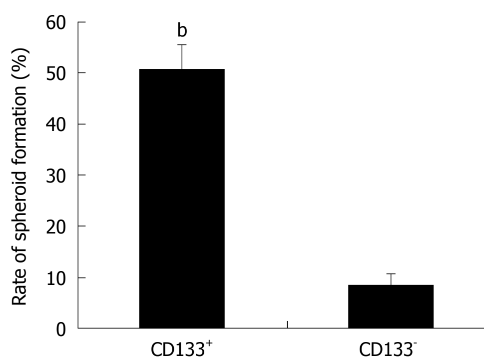


Figure 5 Spheroid-colony-formation assay of CD133+ cells. Sphere-forming cells were isolated by fluorescence-activated cell sorting for marker CD133 and cultured in 96-well plates in serum-free medium (10 cells per well) containing human epidermal growth factor (20 ng/mL) and basic fibroblast growth factor (10 ng/mL). After 2 wk, the total number of spheroid colonies containing more than 20 cells was counted. CD133+ cells generated more spheroid colonies. ^bP < 0.01.

Table 1 Tumorigenicity of CD133+ and CD133- cells sorted from primary gallbladder carcinomas or spheroids

Cell type	Fraction	1st mo	2nd mo	3rd mo^a
Spheroid cells	CD133+	1/6	3/6	6/6
	CD133-	0/6	0/6	1/6
Tumor 3	CD133+	0/3	1/3	3/3
	CD133-	0/3	0/3	1/3
Tumor 4	CD133+	1/3	2/3	3/3
	CD133-	0/3	0/3	0/3

Freshly-sorted cells were injected subcutaneously into the abdominal regions of nude mice at a dose of 10000 cells. ^aP < 0.05.

ent at relatively low percentages in samples from both primary tumors (3.79% in tumor 1 and 3.15% in tumor 2). The CD133+ populations, however, were significantly increased to 18.65% (tumor 1) and 21.54% (tumor 2) in the tumor spheres (P < 0.05, Figure 4). These results suggest that CD133 could be a candidate cellular surface marker for GBC progenitors.

CD133+ GBC cells showed higher spheroid-colony-forming ability in vitro

The growth of spherical colonies is considered to reflect the self-renewal ability and phenotype of CSCs[16]. CD133+ cells were isolated from spheres and placed into stem-cell-selective conditions. After *in vitro* culture for 2 wk, the total number of spheroid colonies containing more than 20 cells was counted, and CD133+ cells generated more spheroid colonies than the CD133- fractions (P < 0.01, Figure 5). These results suggest that the CD133+ subset plays a dominant role in the spheroids.

CD133+ GBC cells showed higher tumorigenicity in vivo

To authenticate the *in vitro* findings, sorted GBC cells were transplanted into nude mice. An apparent difference in tumorigenicity was observed between the cell populations (P < 0.05, Table 1, Figure 6A). It was found that 10^4 CD133+ GBC cells were able to generate tumors in six out of six or three out of three nude mice after 9-12 wk, while the same number of CD133- cells induced tumors in only one out of six or one out of three nude mice, with smaller mass and longer latency. HE staining and immunohistochemistry demonstrated that the xenografts in the immunodeficient mice were generated from the injected human GBC cells. The xenograft tumors revealed similar histologic characteristics and expression of CA19-9 to those of the primary GBC (Figure 6B). Taken together, these results indicate that CD133+ GBC cells exhibited cancer stem-cell-like characteristics, strongly supporting the existence of tumor-initiating cells in this population.

DISCUSSION

A number of studies have demonstrated the presence of CSCs in solid tumors. These cells possess the abilities of self-renewal and differentiation, high tumorigenicity, and resistance to current treatments[10-15]. In this study, we described the characterization of CSCs in human GBC. Previous studies showed that tumor spheres could be generated from tumor cells in serum-free medium and that the constituent cells exhibited the properties of CSCs, including self-renewal, differentiation potential, chemotherapy resistance, and high tumorigenicity[11,14].

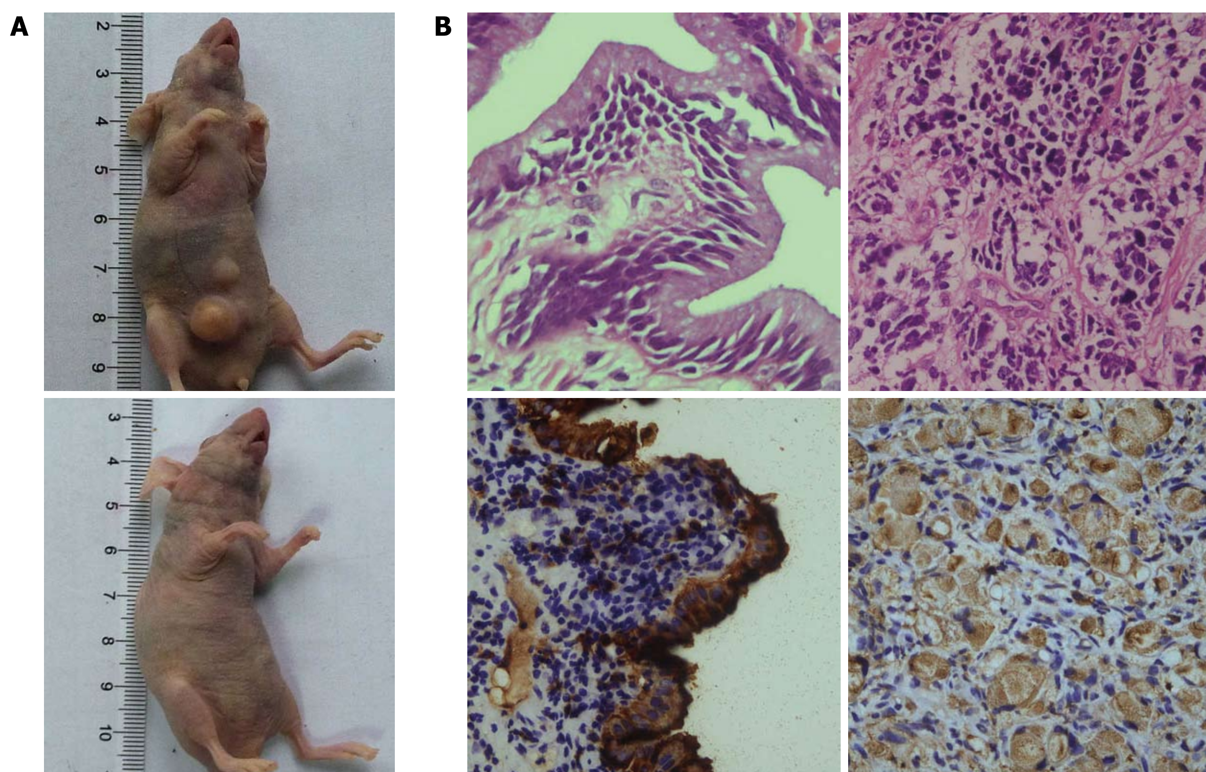


Figure 6 Xenograft formation of CD133+ gallbladder carcinoma cells. Sorted cells were injected subcutaneously into the abdominal regions of nude mice at a dose of 10 000 cells. A: CD133+ cells produced palpable xenograft tumors at the injection site, whereas CD133- cells generated no tumors; B: Hematoxylin and eosin staining of xenograft and expression of CA19-9 (original magnification × 200).

In our experiments, primary GBC cells formed tumor spheres when cultivated under stem-cell-selective conditions similar to those reported previously. The self-renewal and differentiation potentials, proliferation ability and chemosensitivity of the sphere-forming cells were assessed. These cells displayed CSC properties by regenerating new tumor spheres in serum-free medium, overexpressing stem cell markers and showing a higher resistance to chemotherapeutic reagents, while these features were diminished under differentiating conditions. These results indicate that CSCs were enriched in these floating GBC spheres.

Cell surface markers of CSCs can help distinguish, isolate and purify these tumor-initiating cells for further biological investigation. The protein CD133 is cell surface marker for CSCs in brain tumor[11], Ewing's sarcoma[15] and liver cancers[26]. The development and differentiation of human bile ducts and liver are closely related; both start from hepatic endodermal cells and hepatoblasts just after liver primordium formation. We therefore selected CD133 as a potential CSC marker in the current study, and detected its expression in primary GBC and in sphere-forming cells. CD133+ cells comprised a small fraction of the total tumor population in all three samples studied, but represented an increased percentage of the sphere-forming cells. This suggests that CD133 could act as a cell surface marker for CSCs in GBC. We also investigated the use of this cell surface protein as a candidate marker to further identify the CSC phenotype in GBC. The self-renewal ability of CD133+ cells was tested using spheroid-forming assays in serum-free medium. CD133+ cells possessed higher clonogenicity than their antigen-negative counterparts. Subsequent *in vivo* tumorigenesis experiments demonstrated that CD133+ cells possessed higher tumorigenicity than the CD133- subpopulation. Furthermore, the tumors generated in nude mice displayed the same phenotype as the primary GBC tissue. Taken together, these results firmly suggest that CD133+ cells possess the potentials for self-renewal and high tumorigenicity, exhibiting cancer stem-cell-like characteristics in human GBC.

The internal relationship between the expression of CD133 and the characteristics of CSCs remains unclear. Previous studies suggested that CD133 expression was associated with cell motility in melanoma[27] and colorectal cancer cells[28], and a high level of CD133 was also associated with increased resistance to staurosporine-inducing apoptosis[28]. These associations may be due to the interaction between CD133 and the canonical Wnt pathway[27]. However, the role of CD133 in these biological activities remains to be further clarified.

In summary, the results of this study demonstrate that CSCs are enriched in non-adherent spheres derived from GBC cells, and that CD133 protein may represent a cell surface marker for this cell population.

ACKNOWLEDGMENTS

We thank Ying Xiang from Organ Transplantation Institute of Tongji Hospital, Tongji Medical College, Hua-

zhong University of Science and Technology, Wuhan, China, for her technical assistance.

COMMENTS

Background

Gallbladder carcinoma (GBC) is the most common malignant neoplasm of the biliary tract and the seventh most common gastrointestinal cancer. Emerging evidence has shown that the abilities for tumor growth and propagation reside in a small population of tumor cells, termed cancer stem cells (CSCs) or tumor-initiating cells.

Research frontiers

Tumor-initiating cells with distinct cell surface markers have recently been identified in various solid tumors. In this study, the authors demonstrate that primary human GBC cells also contain tumor-initiating cells and that CD133 protein may be a cell surface marker for this cell population.

Innovations and breakthroughs

Previous studies have suggested that CD133 expression is associated with cell motility and is a cell surface marker for tumor-initiating cells in some tumors. This is the first study to report upregulation of CD133 in spheroids derived from primary human GBC cells. Furthermore, the *in vitro* and *in vivo* studies suggest that CD133 protein may represent a cell surface marker for CSCs in GBC.

Applications

This study may provide a novel approach to the diagnosis and treatment of GBC.

Peer review

The presented manuscript deals with extremely thrilling issue of cancer development. It should be of great interest for the readers reflecting progress in our comprehension of carcinogenesis.

REFERENCES

1 **Reid KM**, Ramos-De la Medina A, Donohue JH. Diagnosis and surgical management of gallbladder cancer: a review. *J Gastrointest Surg* 2007; **11**: 671-681

2 **Levy AD**, Murakata LA, Rohrmann CA. Gallbladder carcinoma: radiologic-pathologic correlation. *Radiographics* 2001; **21**: 295-314; questionnaire, 549-555

3 **Roa I**, de Aretxabala X, Araya JC, Villaseca M, Roa J, Guzmán P. [Incipient gallbladder carcinoma. Clinical and pathological study and prognosis in 196 cases]. *Rev Med Chil* 2001; **129**: 1113-1120

4 **Reya T**, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001; **414**: 105-111

5 **Scadden DT**. Cancer stem cells refined. *Nat Immunol* 2004; **5**: 701-703

6 **Wicha MS**, Liu S, Dontu G. Cancer stem cells: an old idea--a paradigm shift. *Cancer Res* 2006; **66**: 1883-1890; discussion 1895-1896

7 **O'Brien CA**, Kreso A, Dick JE. Cancer stem cells in solid tumors: an overview. *Semin Radiat Oncol* 2009; **19**: 71-77

8 **Milas L**, Hittelman WN. Cancer stem cells and tumor response to therapy: current problems and future prospects. *Semin Radiat Oncol* 2009; **19**: 96-105

9 **Bonnet D**, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 1997; **3**: 730-737

10 **Al-Hajj M**, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 2003; **100**: 3983-3988

11 **Singh SK**, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB. Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003; **63**: 5821-5828

12 **Collins AT**, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* 2005; **65**: 10946-10951

13 **Li C**, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM. Identification of pancreatic cancer stem cells. *Cancer Res* 2007; **67**: 1030-1037

14 **Zhang S**, Balch C, Chan MW, Lai HC, Matei D, Schilder JM, Yan PS, Huang TH, Nephew KP. Identification and characterization of ovarian cancer-initiating cells from primary human tumors. *Cancer Res* 2008; **68**: 4311-4320

15 **Suvà ML**, Riggi N, Stehle JC, Baumer K, Tercier S, Joseph JM, Suvà D, Clément V, Provero P, Cironi L, Osterheld MC, Guillou L, Stamenkovic I. Identification of cancer stem cells in Ewing's sarcoma. *Cancer Res* 2009; **69**: 1776-1781

16 **Ailles LE**, Weissman IL. Cancer stem cells in solid tumors. *Curr Opin Biotechnol* 2007; **18**: 460-466

17 **Vermeulen L**, Sprick MR, Kemper K, Stassi G, Medema JP. Cancer stem cells--old concepts, new insights. *Cell Death Differ* 2008; **15**: 947-958

18 **Ricci-Vitiani L**, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, De Maria R. Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007; **445**: 111-115

19 **Wilson H**, Huelsmeyer M, Chun R, Young KM, Friedrichs K, Argyle DJ. Isolation and characterisation of cancer stem cells from canine osteosarcoma. *Vet J* 2008; **175**: 69-75

20 **Agrama HA**, Houssin SF, Tarek MA. Cloning of AFLP markers linked to resistance to Peronosclerospora sorghi in maize. *Mol Genet Genomics* 2002; **267**: 814-819

21 **Fang D**, Nguyen TK, Leishear K, Finko R, Kulp AN, Hotz S, Van Belle PA, Xu X, Elder DE, Herlyn M. A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer Res* 2005; **65**: 9328-9337

22 **Loh YH**, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, Wong KY, Sung KW, Lee CW, Zhao XD, Chiu KP, Lipovich L, Kuznetsov VA, Robson P, Stanton LW, Wei CL, Ruan Y, Lim B, Ng HH. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet* 2006; **38**: 431-440

23 **Wiese C**, Rolletschek A, Kania G, Blyszczuk P, Tarasov KV, Tarasova Y, Wersto RP, Boheler KR, Wobus AM. Nestin expression--a property of multi-lineage progenitor cells? *Cell Mol Life Sci* 2004; **61**: 2510-2522

24 **Vargo-Gogola T**, Rosen JM. Modelling breast cancer: one size does not fit all. *Nat Rev Cancer* 2007; **7**: 659-672

25 **Takaishi S**, Okumura T, Tu S, Wang SS, Shibata W, Vigneshwaran R, Gordon SA, Shimada Y, Wang TC. Identification of gastric cancer stem cells using the cell surface marker CD44. *Stem Cells* 2009; **27**: 1006-1020

26 **Zhu Z**, Hao X, Yan M, Yao M, Ge C, Gu J, Li J. Cancer stem/progenitor cells are highly enriched in CD133+CD44+ population in hepatocellular carcinoma. *Int J Cancer* 2010; **126**: 2067-2078

27 **Rappa G**, Fodstad O, Lorico A. The stem cell-associated antigen CD133 (Prominin-1) is a molecular therapeutic target for metastatic melanoma. *Stem Cells* 2008; **26**: 3008-3017

28 **Elsaba TM**, Martinez-Pomares L, Robins AR, Crook S, Seth R, Jackson D, McCart A, Silver AR, Tomlinson IP, Ilyas M. The stem cell marker CD133 associates with enhanced colony formation and cell motility in colorectal cancer. *PLoS One* 2010; **5**: e10714

S- Editor Tian L **L- Editor** Ma JY **E- Editor** Zheng XM

WJG World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2972

World J Gastroenterol 2011 June 28; 17(24): 2972-2975
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2011 Baishideng. All rights reserved.

CASE REPORT

Pseudopneumoperitoneum in chronic intestinal pseudo-obstruction: A case report

Luigi Camera, Milena Calabrese, Giovanni Sarnelli, Margaret Longobardi, Alba Rocco, Rosario Cuomo, Marco Salvatore

Luigi Camera, Milena Calabrese, Margaret Longobardi, Marco Salvatore, Department of Bio-morphological and Functional Sciences, University "Federico II", 80131 Naples, Italy
Luigi Camera, Institute of Bioimages and Biostructures, National Research Council, 80131 Naples, Italy
Giovanni Sarnelli, Alba Rocco, Rosario Cuomo, Department of Clinical and Experimental Medicine, Gastroenterology Unit, University "Federico II", 80131 Naples, Italy
Author contributions: Camera L contributed case observation and manuscript revision; Calabrese M was involved in manuscript preparation; Sarnelli G conducted anorectal manometry; Longobardi M performed the literature search; Rocco A performed pancolonosopy; Cuomo R and Salvatore M made a major contribution to manuscript editing.
Correspondence to: **Luigi Camera, MD,** Department of Bio-morphological and Functional Sciences, University "Federico II", Via S. Pansini 5, 80131 Naples, Italy. camera@unina.it
Telephone: +39-81-7463560 **Fax:** +39-81-5457081
Received: December 4, 2010 **Revised:** February 9, 2011
Accepted: February 16, 2011
Published online: June 28, 2011

Abstract

Chronic intestinal pseudo-obstruction (CIPO) is a rare disease due to a severe gastrointestinal motility disorder which may mimic, on both clinical and radiological grounds, mechanical obstruction. We report a case of a 26-year-old woman who presented to our institution for plain abdominal radiography for referred long-lasting constipation with recurrent episodes of abdominal pain and distension. At X-ray, performed both in the upright and supine position, an isolated air-fluid level was depicted in the left flank, together with a number of radiological signs suggestive of pneumoperitoneum. First, subphrenic radiolucency could be observed in the upright film. Second, the intestinal wall of some jejunal loops appeared to be outlined in the right flank. Third, the inferior cardiac border was clearly depicted in the upright film. The patient however had no evidence of peritoneal signs but only hypoactive bowel movements. Unenhanced multi-detector computed tomography (MDCT) of the abdomen and pelvis was therefore performed. MDCT revealed abnormal air-driven distension of the small and large bowel, without evidence of extraluminal air. All radiological signs of pneumoperitoneum turned out to be false-positive results. The patient was submitted to pan-colonoscopy and to anorectal manometry to rule out Hirshprung's disease, and was finally discharged with a diagnosis of CIPO.

© 2011 Baishideng. All rights reserved.

Key words: Pseudopneumoperitoneum; Abdominal radiography; Multi-detector computed tomography; Motility disorders; Chronic intestinal pseudo-obstruction

Peer reviewer: Andrew Seng Boon Chua, MD, Department of Gastroenterology, Gastro Centre Ipoh, 1, lorong Rani, 31, lebuhraya Tmn Ipoh, Ipoh Garden South, IPOH 30350, Malaysia

Camera L, Calabrese M, Sarnelli G, Longobardi M, Rocco A, Cuomo R, Salvatore M. Pseudopneumoperitoneum in chronic intestinal pseudo-obstruction: A case report. *World J Gastroenterol* 2011; 17(24): 2972-2975 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2972.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2972

INTRODUCTION

Chronic intestinal pseudo-obstruction (CIPO) is a rare motility disorder of the gastrointestinal (GI) tract that is usually observed in a number of different neuropathies, mesenchymopathies or myopathies, but it can also be idiopathic[1]. It is characterized by failure of the GI tract to propel its content and may result in a clinical picture mimicking mechanical obstruction, with patients complaining of recurrent episodes of abdominal distension,

with or without abdominal pain, nausea and vomiting[2].

The diagnosis of CIPO is mainly clinical and is usually postulated after exclusion of any organic lesion occluding the gut lumen at endoscopy, while it can be supported by radiographic documentation of dilated small and/or large bowel loops, without evidence of a definite transition zone. This, in adults, is now best accomplished by multi-detector computed tomography (MDCT), which can obviate the need for unnecessary laparotomy[3]. However, plain abdominal films are one of the most important examinations in the diagnosis of CIPO in which abnormal air-driven distension of both small and large bowel is usually depicted[4].

Here, we report a case of a 26-year-old woman who presented to our institution for plain abdominal radiography as part of clinical and radiological work-up for referred long-lasting constipation with recurrent episodes of abdominal pain and distension. At X-ray, an isolated air-fluid level could be observed in the left flank region, together with a number of radiological signs suggestive of pneumoperitoneum. These could be appreciated both in the upright and supine films, but turned out to be false-positive results, as an unenhanced MDCT scan failed to show evidence of extra-luminal air in the peritoneal cavity.

CASE REPORT

A 26-year-old woman with a history of recurrent episodes of abdominal pain and distension came to our institution to undergo plain abdominal radiography. Abdominal plain films were obtained both in the upright (Figure 1A) and supine (Figure 1B) position. The upright film showed an isolated air-fluid level in the left flank region, consistent with an obstruction of the descending colon, together with a number of X-ray findings suggestive of pneumoperitoneum. First, subphrenic radiolucencies were clearly depicted on both sides in the upright film. Second, the inferior cardiac border could be observed from the cardiac apex to the inferior vena cava. Third, the intestinal wall of some jejunal loops appeared to be outlined in the right flank (Figure 1A). This latter finding was also evident in the supine film, configuring the so-called bas-relief or Rigler's sign together with the lack of normal hepatic shadow in the right subphrenic space, the so-called hyperlucent liver sign (Figure 1B). Based on these X-ray findings, the on-call radiologist immediately contacted the referring physician to communicate a diagnosis of pneumoperitoneum.

At physical examination, however, only a large abdominal distension with hypoactive bowel could be observed, but no peritoneal signs. It was therefore agreed upon to perform MDCT (Aquilion 4; Toshiba, Japan). This was performed with a detector configuration of 4 × 5 mm, table speed 30 mm/s, rotation time = 0.5 s, beam pitch = 0.75, 120 kVp, 250 mA. Only unenhanced acquisition was performed. The MDCT scan showed marked dilation of the large bowel, with both hepatic and splenic flexures displaced underneath the diaphragm (Figure 2A). Air-driven distension involved both small (Figure 2B) and large bowel (Figure 2C) loops. There was no evidence of any obstructive lesion and both the sigmoid colon and rectum were normally filled with feces (Figure 2D). No evidence of free air in the peritoneal cavity was found, therefore, a diagnosis of pneumoperitoneum could not be confirmed.

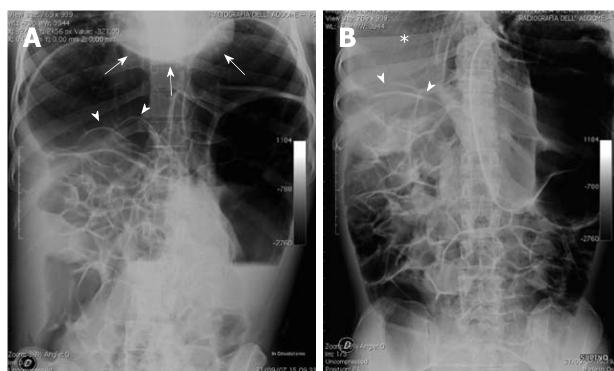


Figure 1 Upright (A) and supine (B) films in a female patient complaining of long-lasting constipation, with recurrent episodes of abdominal pain and distension. A: An isolated air-fluid level is depicted in the left flank, suggesting mechanical obstruction at the level of the descending colon. Subphrenic radiolucency is evident on both sides, together with the outlining of the intestinal wall of some small bowel loops in the right flank (arrowheads) and that of the inferior cardiac border (arrows); B: Outline of the intestinal wall of small bowel loops appears to be depicted in the right flank (arrowheads), configuring the so-called bas-relief or Rigler's sign. In addition, hyperlucency is depicted in the right subphrenic space in place of the normal hepatic shadow, configuring the bright or hyperlucent liver sign (*).

Further clinical, biochemical and instrumental investigation involving pan-colonoscopy and anorectal manometry revealed no underlying causes, and the patient was finally discharged with a diagnosis of CIPO.

DISCUSSION

Pneumoperitoneum is usually referred to as the presence of free air within the peritoneal cavity. Its radiological diagnosis usually relies on the evidence of typical subphrenic radiolucency in the posterior-anterior projection taken in the upright position[5]. As a result, the term pseudopneumoperitoneum is used when the subphrenic radiolucency does not correspond to free intraperitoneal air[6], but can be traced to either subphrenic fat pad[7] or basal lung atelectasis[8]. In these cases, the clarifying role of CT has been acknowledged[9].

To the best of our knowledge, whereas the possible occurrence of pneumoperitoneum in CIPO has been described[10], the occurrence of pseudopneumoperitoneum in CIPO has not been reported to date. In our case, subphrenic radiolucency depicted in the upright film (Figure 1A) was due to hepatic and splenic colonic flexures abnormally dilated and displaced below the diaphragmatic shadows, as shown by CT (Figure 2A).

In the emergency setting, prompt recognition of radiological signs of pneumoperitoneum in the anterior-

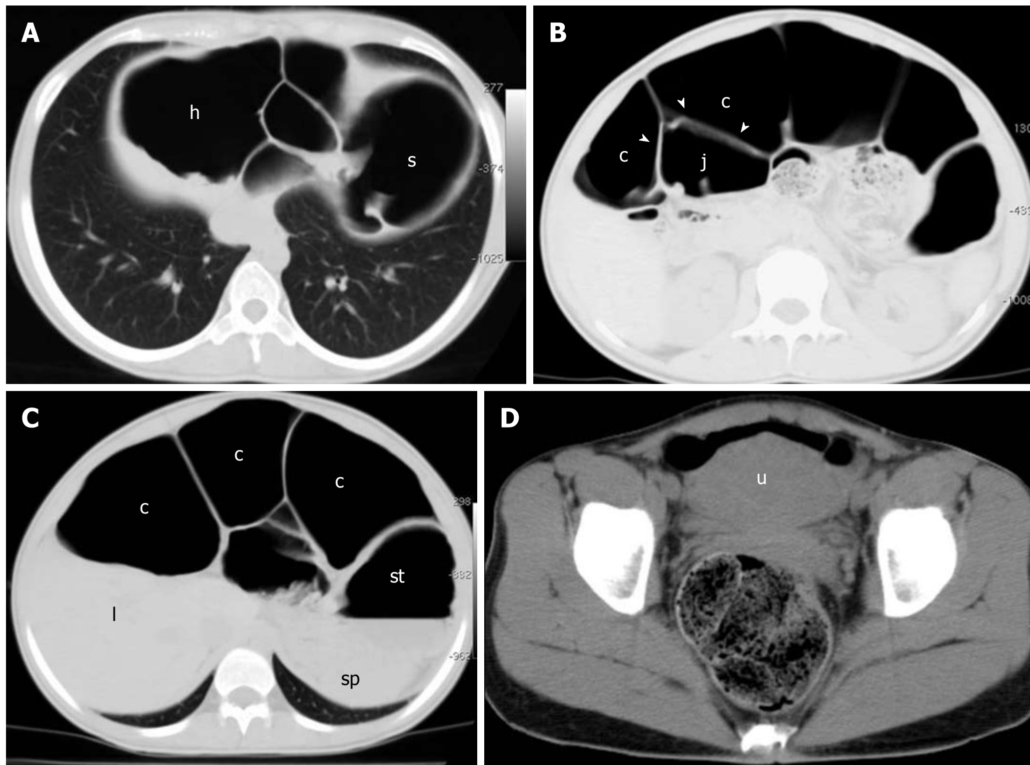


Figure 2 Unenhanced four-row MDCT. Images are displayed with lung (A-C) and soft tissue (D) windows. A: Hepatic (h) and splenic (s) flexures, air-filled and abnormally dilated, are displaced under the diaphragm, accounting for the subphrenic radiolucency depicted in the upright film; B: Intraluminal air in the large bowel (c) appears to outline the intestinal wall (arrowheads) of a jejunal loop (j) that is also mildly dilated and air-filled, accounting for the Rigler's sign depicted on the upright and supine films; C: Abnormally dilated colonic segment situated between the liver (l) and anterior abdominal wall, simulating the hyperlucent (bright) liver sign depicted in the supine film; st: Stomach; sp: Spleen; D: Rectum is normally filled with feces. u: Uterus.

posterior projection taken in the supine position may be of diagnostic value because it allows a confident diagnosis of pneumoperitoneum whenever an L-L projection is not available or cannot be performed[11].

These radiological signs, which can be mostly traced to either the intestinal wall[12] or various peritoneal folds[13-15], outlined by the presence of free air within the peritoneal cavity, have been recently revised and classified into four major categories as bowel-related signs, right upper quadrant signs, peritoneal ligament signs, and other signs[16].

For at least one of these signs, namely the Rigler's sign, the possible occurrence of false-positive cases is well acknowledged. Rigler's sign can be simulated by two contiguous, moderately dilated and air-filled loops of bowel, whereby intraluminal air in one loop of bowel may appear to outline the wall of the adjacent loop[17]. These false-positives cases, however, have only been reported in the supine film where the sign itself was originally described[12]. To the best of our knowledge, the occurrence of a false-positive Rigler's sign in the upright position has never been described. As shown by MDCT, the intestinal wall of some jejunal loops was in our case outlined by intraluminal air in the large bowel, which was abnormally dilated (Figure 2B). The anomalous dislocation of jejunal loops in the right flank was not further investigated, although it was likely due to concomitant malrotation.

Possible occurrence of false-positive results for the inferior cardiac border sign were also postulated in the original description. The inferior border of the heart becomes visible on supine radiography whenever it is outlined by air; either free air in the peritoneal cavity, such as in pneumoperitoneum, or air within the pleural and/or pericardial sac. Occasionally, gas-filled loops collected beneath the left hemi-diaphragm may simulate this sign[18]. As with Rigler's sign, these false-positive signs are also expected to occur in the supine position and not in the upright. In our case, however, the sign could not be detected in the supine film because of suboptimal technical positioning (Figure 1B).

Finally, the hyperlucent liver sign which could be observed on the supine film (Figure 1B) also turned out to be a false-positive result because it was clearly due to the interposition of dilated, air-filled large bowel loops between the liver and the anterior abdominal wall, as shown by MDCT (Figure 2C). To the best of our knowledge, false-positive cases of hyperlucent liver sign have never been reported.

In the present case, MDCT not only ruled out the radiological diagnosis of pneumoperitoneum but it also excluded mechanical obstruction, and showed that the sigmoid colon and rectum were normally filled with feces, without evidence of a transition zone (Figure 2D). As this latter CT finding is rather atypical in patients with Hirschsprung's disease[19], this was also excluded by anorectal manometry, which revealed a normal inhibitory

rectoanal reflex[20]. The patient was therefore discharged with a diagnosis of CIPO.

We have described a case of pseudopneumoperitoneum in a patient with CIPO. All radiological findings of pneumoperitoneum that were clearly depicted in the upright and supine films turned out to be false-positive results, as shown by MDCT. To the best of our knowledge, such a case has not been reported to date.

REFERENCES

1 **De Giorgio R**, Sarnelli G, Corinaldesi R, Stanghellini V. Advances in our understanding of the pathology of chronic intestinal pseudo-obstruction. *Gut* 2004; **53**: 1549-1552

2 **Stanghellini V**, Cogliandro RF, De Giorgio R, Barbara G, Morselli-Labate AM, Cogliandro L, Corinaldesi R. Natural history of chronic idiopathic intestinal pseudo-obstruction in adults: a single center study. *Clin Gastroenterol Hepatol* 2005; **3**: 449-458

3 **Merlin A**, Soyer P, Boudiaf M, Hamzi L, Rymer R. Chronic intestinal pseudo-obstruction in adult patients: multidetector row helical CT features. *Eur Radiol* 2008; **18**: 1587-1595

4 **Antonucci A**, Fronzoni L, Cogliandro L, Cogliandro RF, Caputo C, De Giorgio R, Pallotti F, Barbara G, Corinaldesi R, Stanghellini V. Chronic intestinal pseudo-obstruction. *World J Gastroenterol* 2008; **14**: 2953-2961

5 **Miller RE**. The radiological evaluation of intraperitoneal gas (pneumoperitoneum). *CRC Crit Rev Clin Radiol Nucl Med* 1973; **4**: 61-85

6 **Mokrohisky JF**. Pseudopneumoperitoneum; simulated free air in the peritoneal cavity. *Am J Roentgenol Radium Ther Nucl Med* 1958; **79**: 293-300

7 **Fisher MS**. The simulation of pneumoperitoneum by basal atelectasis. *Br J Radiol* 1968; **41**: 701

8 **Fataar S**, Schulman A. Pseudopneumoperitoneum due to subphrenic fat. *AJR Am J Roentgenol* 1981; **137**: 391-392

9 **Nseif M**, Berger A, Bely N, Zinzindohoue F, Cellier C, Frija G, Cugnenc P. False radiologic pneumoperitoneum. The value of emergency abdominal computed tomography. *J Chir* (Paris) 1997; **134**: 329-331

10 **Kim HW**, Chon NR, Kim YS, Kim JH, Park H. A case of spontaneous pneumoperitoneum associated with idiopathic intestinal pseudoobstruction. *Korean J Gastroenterol* 2009; **54**: 395-398

11 **Levine MS**, Scheiner JD, Rubesin SE, Laufer I, Herlinger H. Diagnosis of pneumoperitoneum on supine abdominal radiographs. *AJR Am J Roentgenol* 1991; **156**: 731-735

12 **Rigler LG**. Spontaneous pneumoperitoneum: A roentgenologic sign found in the supine position. *Radiology* 1941; **37**: 604-607

13 **Weiner CI**, Diaconis JN, Dennis JM. The "inverted V": a new sign of pneumoperitoneum. *Radiology* 1973; **107**: 47-48

14 **Cho KC**, Baker SR. Visualization of the extrahepatic segment of the ligamentum teres: a sign of free air on plain radiographs. *Radiology* 1997; **202**: 651-654

15 **Grassi R**, Catalano O, Pinto A, Fanucci A, Rotondo A, Di Mizio R. Case report: identification of the transverse mesocolon and root of small bowel mesentery; a new sign of pneumoperitoneum. *Br J Radiol* 1996; **69**: 774-776

16 **Chiu YH**, Chen JD, Tiu CM, Chou YH, Yen DH, Huang CI, Chang CY. Reappraisal of radiographic signs of pneumoperitoneum at emergency department. *Am J Emerg Med* 2009; **27**: 320-327

17 **Ly JQ**. The Rigler sign. *Radiology* 2003; **228**: 706-707

18 **Klein DL**. Visibility of the inferior heart border in pneumoperitoneum. *AJR Am J Roentgenol* 1981; **137**: 622-623

19 **Kim HJ**, Kim AY, Lee CW, Yu CS, Kim JS, Kim PN, Lee MG, Ha HK. Hirschsprung disease and hypoganglionosis in adults: radiologic findings and differentiation. *Radiology* 2008; **247**: 428-434

20 **Morais MB**, Sdepanian VL, Tahan S, Goshima S, Soares AC, Motta ME, Fagundes Neto U. Effectiveness of anorectal manometry using the balloon method to identify the inhibitory recto-anal reflex for diagnosis of Hirschsprung's disease. *Rev Assoc Med Bras* 2005; **51**: 313-317

S- Editor Tian L **L- Editor** Kerr C **E- Editor** Zheng XM

WJG World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i24.2976

World J Gastroenterol 2011 June 28; 17(24): 2976
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2011 Baishideng. All rights reserved.

LETTERS TO THE EDITOR

Hepatocellular carcinoma and industrial epidemics

Alain Braillon, Gérard Dubois

Alain Braillon, Gres, 27, rue Voiture, 80000 Amiens, France
Gérard Dubois, Public Health, Northern hospital, place Victor Pauchet, 80000 Amiens, France
Author contributions: Braillon A and Dubois G conceived, analyzed and wrote the letter.
Supported by Comité départemental de l'Oise de la Ligue Contre le Cancer
Correspondence to: Dr. Alain Braillon, Gres, 27, rue Voiture, 80000 Amiens, France. braillon.alain@gmail.com
Telephone: +33-3-22955539
Received: September 1, 2010 Revised: January 17, 2011
Accepted: January 24, 2011
Published online: June 28, 2011

Abstract

Worldwide, the burden of the non viral causes of hepatocellular carcinoma (HCC) is usually underestimated. Clearly industrial goods, tobacco, alcohol and processed foods are the agents of new epidemics in modern times which far outscore the burden of infectious agents on morbidity and mortality. Smoking, a dose-related contributing factor for HCC, receives too little attention in clinical practice. In France, tobacco, hepatitis B and C virus and alcohol are the main risk factors for HCC mortality (33%, 31% and 26%, respectively). In developing countries, where tobacco consumption is dramatically increasing, this epidemic may soon surpass hepatitis B. Obesity and diabetes are the contributing factors too. The role of industrial processed foods in the increase of the prevalence of obesity and diabetes cannot be ignored.

© 2011 Baishideng. All rights reserved.

Key words: Hepatocellular carcinoma; Tobacco; Alcohol; Processed foods; Industrial epidemics

Peer reviewer: Dr. Ajith TA, Associate Professor, Department of Biochemistry, Amala Institute of Medical Sciences, Amala Nagar, Thrisssur, Kerala-680 555, Pakistan

Braillon A, Dubois G. Hepatocellular carcinoma and industrial epidemics. *World J Gastroenterol* 2011; 17(24): 2976 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i24/2976.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i24.2976

TO THE EDITOR

Worldwide, hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer-related death. The Journal must be acknowledged for the publication of Blonski *et al*[1] review who wisely underlined the burden of non viral causes of HCC which are usually underestimated.

Blonski *et al*[1] rightly stressed the role of smoking, a dose related contributing factor for HCC, and this is important because some are still ignoring it[2]. In France, tobacco, hepatitis B and C virus and alcohol are the main risk factors for HCC mortality (33%, 31% and 26%, respectively)[3,4].

Blonski *et al*[1] also listed obesity and diabetes as contributing factors for HCC. The role of industrial processed foods in the increase of the prevalence of obesity and diabetes cannot be ignored.

Clearly industrial goods, tobacco, alcohol and processed foods are the agents of new epidemics in modern times which far outscore the burden of infectious agents on morbidity and mortality.

REFERENCES

1 **Blonski W**, Kotlyar DS, Forde KA. Non-viral causes of hepatocellular carcinoma. *World J Gastroenterol* 2010; **16**: 3603-3615
2 **Kumagi T**, Hiasa Y, Hirschfield GM. Hepatocellular carcinoma for the non-specialist. *BMJ* 2009; **339**: b5039
3 **Lee YC**, Cohet C, Yang YC, Stayner L, Hashibe M, Straif K. Meta-analysis of epidemiologic studies on cigarette smoking and liver cancer. *Int J Epidemiol* 2009; **38**: 1497-1511
4 **Dubois G**, Braillon A. Hepatocellular carcinoma: again, tobacco is the first enemy. *Int J Epidemiol* 2010; **39**: 1399

S- Editor Sun H **L- Editor** Wang XL **E- Editor** Zheng XM

WJG World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2011 June 28; 17(24): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2011 Baishideng. All rights reserved.

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

Tomoharu Yoshizumi, MD, PhD, Department of Surgery, Saiseikai Fukuoka General Hospital, 1-3-46, Tenjin, Chuou-ku, Fukuoka, 810-0001, Japan

Boris Kirshtein, MD, Department of Surgery "A" , Soroka Medical Center, Ben Gurion University of the Negev, POB 151, Beer Sheva, 84101, Israel

Shoichiro Sumi, MD, PhD, Associate Professor, Department of Organ Reconstruction, Institute for Frontier Medical Sciences, Kyoto University, Sakyo-ku, Kyoto, 606-8507, Japan

George Y Wu, Professor, Department of Medicine, Division of Gastroenterology-Hepatology, University of Connecticut Health Center, 263 Farmington Ave, Farmington, CT 06030, United States

Herwig R Cerwenka, Dr., Professor, Department of Surgery, Medical University of Graz, Auenbruggerplatz 29, A-8036 Graz, Austria

Philip Abraham, Dr., Professor, Consultant Gastroenterologist and Hepatologist, P. D. Hinduja National Hospital and Medical Research Centre, Veer Savarkar Marg, Mahim, Mumbai 400 016, India

Rami Moucari, MD, PhD, Department Of Internal Medicine, Belle Vue Medical Center, Saint Joseph University, Beirut 295, Lebanon

Ali Canbay, MD, Professor of Medicine, University Hospital Essen, Hufelandstr. 55, D-45122 Essen, Germany

Dina G Tiniakos, MD, PhD, Associate Professor, Laboratory of Histology and Embryology, Medical School University of Athens, 75, M. Asias str, Goudi, Athens 11527, Greece

Mitsuo Shimada, Professor, Department of Digestive and Pediatric Surgery, Tokushima University, Kuramoto 3-18-15, Tokushima 770-8503, Japan

Salvatore Gruttadauria, MD, Assistant Professor, Abdominal Transplant Surgery, ISMETT, Via E. Tricomi, 190127 Palermo, Italy

Zong-Jie Cui, PhD, Professor, Institute of Cell Biology, Beijing Normal University, 19 XinJieKouWaiDaJie, Beijing 100875, China

Ian C Lawrance, MB, BS (Hons), PhD, FRACP, Professor, Director, Centre for Inflammatory Bowel Disease, School of Medicine and Pharmacology, University of Western Australia, Centre for Inflammatory Bowel Disease, Fremantle Hospital, T Block, Alma Street, Fremantle WA 6160, Australia

Sri P Misra, Professor, Gastroenterology, Moti Lal Nehru Medical College, Allahabad 211001, India

Francisco Rodriguez-Frias, PhD, Proteins Hepatitis Molecular Genetics Unitat, Biochemistry Department, Vall d'Hebron Unicersitary Hospital, Barcelona 08035, Spain

Tamir Miloh, MD, Associate Professor, Director of Pediatric Liver/Liver Transplantation Program, Division of Gastroenterology, Phoenix Children's Hospital, 1919 E Thomas Rd, Main Building, Second Floor, Phoenix, AZ 85016, United States

Hong Joo Kim, MD, Professor, Department of Internal Medicine, Sungkyunkwan University Kangbuk Samsung Hospital, 108, Pyung-Dong, Jongro-Ku, Seoul 110-746, South Korea

Mark Pines, PhD, Institute of Animal Sciences, Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel

Yujin Hoshida, MD, PhD, Cancer Program, Broad Institute, 7 Cambridge Center, Cambridge, MA 02142, United States

Chanjuan Shi, MD, PhD, Assistant Professor, Department of Pathology, Vanderbilt University , 1161 21st Ave. So, MCN C-2318A, Nashville, TN 37232-2561, United States

Ashok Kumar, MD, Dr., Department of Surgical Gastroenterology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, 226014, India

Itaru Endo, MD, PhD, Professor and Chairman, Department of Gastroenterological Surgery, Yokohama City University, Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama, 2360004, Japan

WJG World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2011 June 28; 17(24): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2011 Baishideng. All rights reserved.

MEETINGS

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2011 June 28; 17(24): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2011 Baishideng. All rights reserved.

INSTRUCTIONS TO AUTHORS

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

Maximization of personal benefits

The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJG* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article via online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJG* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJG* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclusion; and (4) Maximization of the benefits of employees: It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal. We insist on strengthening our team cultivation and construction so that every employee, in an open, fair and transparent environment, could contribute their wisdom to edit and publish high-quality articles, thereby realizing the maximization of the personal benefits of editorial board members, authors and readers, and yielding the greatest social and economic benefits.

Aims and scope

The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

Columns

The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

Name of journal

World Journal of Gastroenterology

ISSN and EISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

Indexed and Abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifer, and Directory of Open Access Journals. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

Published by
Baishideng Publishing Group Co., Limited

SPECIAL STATEMENT

All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

Biostatistical editing

Statisital review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc*. The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

Conflict-of-interest statement

In the interests of transparency and to help reviewers assess any potential bias, *WJG* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: http://www.icmje.org/ethical_4conflicts.html.

Sample wording: [Name of individual] has received fees for serving as a speaker, a consultant and an advisory board member for [names of organizations], and has received research funding from [names of organization]. [Name of individual] is an employee of [name of organization]. [Name of individual] owns stocks and shares in [name of organization]. [Name of individual] owns patent [patent identification and brief description].

Statement of informed consent

Manuscripts should contain a statement to the effect that all human studies have been reviewed by the appropriate ethics committee or it should be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. Authors should also draw attention to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964, as revised in 2004).

Statement of human and animal rights

When reporting the results from experiments, authors should follow the highest standards and the trial should conform to Good Clinical Practice (for example, US Food and Drug Administration Good Clinical Practice in FDA-Regulated Clinical Trials; UK Medicines Research Council Guidelines for Good Clinical Practice in Clinical Trials) and/or the World Medical Association Declaration of Helsinki. Generally, we suggest authors follow the lead investigator's national standard. If doubt exists whether the research was conducted in accordance with the above standards, the authors must explain the rationale for their approach and demonstrate that the institutional review body explicitly approved the doubtful aspects of the study.

Before submitting, authors should make their study approved by the relevant research ethics committee or institutional review board. If human participants were involved, manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and appropriate informed consent of each. Any personal item or information will not be published without explicit consents from the involved patients. If experimental animals were used, the materials and methods (experimental procedures) section must clearly indicate that appropriate measures were taken to minimize pain or discomfort, and details of animal care should be provided.

SUBMISSION OF MANUSCRIPTS

Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of Baishideng Publishing Group Co., Limited, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the ICMJE to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www.clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

Online submissions

Manuscripts should be submitted through the Online Submission

System at: http://www.wjgnet.com/1007-9327office. Authors are highly recommended to consult the ONLINE INSTRUCTIONS TO AUTHORS (http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm) before attempting to submit online. For assistance, authors encountering problems with the Online Submission System may send an email describing the problem to wjg@wjgnet.com, or by telephone: +86-10-5908-0039. If you submit your manuscript online, do not make a postal contribution. Repeated online submission for the same manuscript is strictly prohibited.

MANUSCRIPT PREPARATION

All contributions should be written in English. All articles must be submitted using word-processing software. All submissions must be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Style should conform to our house format. Required information for each of the manuscript sections is as follows:

Title page

Title: Title should be less than 12 words.

Running title: A short running title of less than 6 words should be provided.

Authorship: Authorship credit should be in accordance with the standard proposed by ICMJE, based on (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published. Authors should meet conditions 1, 2, and 3.

Institution: Author names should be given first, then the complete name of institution, city, province and postcode. For example, Xu-Chen Zhang, Li-Xin Mei, Department of Pathology, Chengde Medical College, Chengde 067000, Hebei Province, China. One author may be represented from two institutions, for example, George Sgourakis, Department of General, Visceral, and Transplantation Surgery, Essen 45122, Germany; George Sgourakis, 2nd Surgical Department, Korgialenio-Benakio Red Cross Hospital, Athens 15451, Greece.

Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

Supportive foundations: The complete name and number of supportive foundations should be provided, e.g. Supported by National Natural Science Foundation of China, No. 30224801

Correspondence to: Only one corresponding address should be provided. Author names should be given first, then author title, affiliation, the complete name of institution, city, postcode, province, country, and email. All the letters in the email should be in lower case. A space interval should be inserted between country name and email address. For example, Montgomery Bissell, MD, Professor of Medicine, Chief, Liver Center, Gastroenterology Division, University of California, Box 0538, San Francisco, CA 94143, United States. montgomery.bissell@ucsf.edu

Telephone and fax: Telephone and fax should consist of +, country number, district number and telephone or fax number, e.g. Telephone: +86-10-59080039 Fax: +86-10-85381893

Peer reviewers: All articles received are subject to peer review. Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in *WJG*, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

Abstract

There are unstructured abstracts (no more than 256 words) and structured abstracts (no more than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no more than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections. AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/…"; MATERIALS AND METHODS (no more than 140 words); RESULTS (no more than 294 words): You should present P values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$; CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be

used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A:...; B:...; C:...; D:...; E:...; F:...; G: …*etc*. It is our principle to publish high resolution-figures for the printed and E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc*., and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^aP < 0.05$, $^bP < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^cP < 0.05$ and $^dP < 0.01$ are used. A third series of P values can be expressed as $^eP < 0.05$ and $^fP < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc*., in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

Coding system

The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that...".

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

PMID and DOI

Pleased provide PubMed citation numbers to the reference list, e.g. PMID and DOI, which can be found at http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed and http://www.crossref.org/SimpleTextQuery/, respectively. The numbers will be used in E-version of this journal.

Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315223018.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc*.

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc*.

Biology: *H. pylori*, *E coli*, *etc*.

Examples for paper writing

Editorial: http://www.wjgnet.com/1007-9327/g_info_20100315220036.htm

Frontier: http://www.wjgnet.com/1007-9327/g_info_20100315220305.htm

Topic highlight: http://www.wjgnet.com/1007-9327/g_info_20100315220601.htm

Observation: http://www.wjgnet.com/1007-9327/g_info_20100312232427.htm

Guidelines for basic research: http://www.wjgnet.com/1007-9327/g_info_20100315220730.htm

Guidelines for clinical practice: http://www.wjgnet.com/1007-9327/g_info_20100315221301.htm

Review: http://www.wjgnet.com/1007-9327/g_info_20100315221554.htm

Original articles: http://www.wjgnet.com/1007-9327/g_info_20100315221814.htm

Brief articles: http://www.wjgnet.com/1007-9327/g_info_20100312231400.htm

Case report: http://www.wjgnet.com/1007-9327/g_info_20100315221946.htm

Letters to the editor: http://www.wjgnet.com/1007-9327/g_info_20100315222254.htm

Book reviews: http://www.wjgnet.com/1007-9327/g_info_20100312231947.htm

Guidelines: http://www.wjgnet.com/1007-9327/g_info_20100312232134.htm

RESUBMISSION OF THE REVISED MANUSCRIPTS

Please revise your article according to the revision policies of *WJG*. The revised version includes manuscript and high-resolution image figures. The author should re-submit the revised manuscript online, along with printed high-resolution color or black and white photos; Copyright transfer letter, and responses to the reviewers, and science news are sent to us *via* email.

Editorial Office

World Journal of Gastroenterology

Editorial Department: Room 903, Building D,
Ocean International Center,
No. 62 Dongsihuan Zhonglu,

Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893

Language evaluation

The language of a manuscript will be graded before it is sent for revision. (1) Grade A: priority publishing; (2) Grade B: minor language polishing; (3) Grade C: a great deal of language polishing needed; and (4) Grade D: rejected. Revised articles should reach Grade A or B.

Copyright assignment form

Please download a Copyright assignment form from http://www.wjgnet.com/1007-9327/g_info_20100315222818.htm.

Responses to reviewers

Please revise your article according to the comments/suggestions provided by the reviewers. The format for responses to the reviewers' comments can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315222607.htm.

Proof of financial support

For paper supported by a foundation, authors should provide a copy of the document and serial number of the foundation.

Links to documents related to the manuscript

WJG will be initiating a platform to promote dynamic interactions between the editors, peer reviewers, readers and authors. After a manuscript is published online, links to the PDF version of the submitted manuscript, the peer-reviewers' report and the revised manuscript will be put on-line. Readers can make comments on the peer reviewer's report, authors' responses to peer reviewers, and the revised manuscript. We hope that authors will benefit from this feedback and be able to revise the manuscript accordingly in a timely manner.

Science news releases

Authors of accepted manuscripts are suggested to write a science news item to promote their articles. The news will be released rapidly at EurekAlert/AAAS (http://www.eurekalert.org). The title for news items should be less than 90 characters; the summary should be less than 75 words; and main body less than 500 words. Science news items should be lawful, ethical, and strictly based on your original content with an attractive title and interesting pictures.

Publication fee

WJG is an international, peer-reviewed, Open-Access, online journal. Articles published by this journal are distributed under the terms of the Creative Commons Attribution Non-commercial License, which permits use, distribution, and reproduction in any medium, provided the original work is properly cited, the use is non commercial and is otherwise in compliance with the license. Authors of accepted articles must pay a publication fee. The related standards are as follows. Publication fee: 1300 USD per article. Editorial, topic highlights, book reviews and letters to the editor are published free of charge.